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ABSTRACT

Title of Dissertation: PURIFICATION AND PROPERTIES OF THE EXTRACELLULAR LIPASE, LIP A, FROM *ACINETOBACTER* SP. RAG-1

Erick A. Snellman, Doctor of Philosophy, 2002

Dissertation directed by: Professor Rita R. Colwell
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The major objectives of this research were: (a) understand the temporal and spatial distribution of lipase production by *Acinetobacter* sp. RAG-1 during growth on hexadecane and triglycerides; (b) purify the extracellular lipase; and (c) to examine its role in modifying the fatty acid component of the bioemulsifier, emulsan, produced by this bacterium. To achieve these objectives, the lipase was produced and purified from RAG-1 cells grown on hexadecane and the properties of the protein investigated. The majority of the enzyme was released into the growth medium during transition to stationary phase. The lipase showed high stability in hexadecane medium where it remained active for longer than 48 hr. at 30°C. Therefore, minimal

medium supplemented with 10 mM hexadecane was selected for purification of the lipase. An 8% yield and greater than 10-fold purification were achieved. The protein demonstrated little affinity for anion exchange resins. However, contaminating proteins were removed by passing crude supernatants over a Mono Q column. The lipase was further purified by hydrophobic interaction chromatography, employing a butyl sepharose matrix, and eluted with an increasing Triton X-100 gradient. The protein has an apparent molecular weight of 33 kDa, determined from SDS PAGE. LipA was found to be stable at pH values of 5.8 – 9.0 and showed optimal activity at approximately pH 9.0. The lipase remained highly active at temperatures up to 70°C and showed a 3-fold increase in activity over the standard assay temperature (30°C) at its temperature optimum (55°C). LipA was found to be active against a wide range of fatty acid esters of *p*-nitrophenyl but demonstrated higher activity toward medium length acyl chains (C_6, C_8). The enzyme demonstrated hydrolytic activity toward emulsions of both medium and long chain triglycerides, determined by zymogram. RAG-1 lipase was stabilized by Ca^{+2}. Loss in activity was not observed in preparations containing the cation, whereas 70% loss was observed over a 30-hr period in its absence. The lipase was strongly inhibited by EDTA, Hg^{+2}, and Cu^{+2}, but did not lose activity after incubation with Ca^{+2}, Mg^{+2}, Zn^{+2}, Fe^{+3}, Co^{+2}, and Rb^{+}. The protein retained more than 75% of the initial activity after exposure to most of the organic solvents employed in this study. It was, however, rapidly inactivated by pyridine. LipA was found to be highly active in organic media, as demonstrated by transesterification of vinyl butyrate with 1-octanol. Similar conditions were employed to examine transesterification activity of LipA toward emulsan fatty acids.

However, it could not be conclusively demonstrated that LipA-catalyzed trans-acylation of the polymer occurred using an exogenous *n*-alkanoic acid. RAG-1 lipase was found to increase emulsifying activity of emulsan. Emulsan incubated in the presence of LipA showed a greater than 2-fold increase in emulsifying activity over control experiments containing emulsan and selected proteins. A model was proposed to show LipA as an emulsan-associated protein.

DEDICATION

This dissertation is dedicated with love and gratitude to my wife, [redacted], and my daughter, Abby

ACKNOWLEDGEMENTS

I wish to express my gratitude to my advisor, Dr. Rita R. Colwell, for her encouragement and support during the course of this work. Her unfailing enthusiasm for my work and her belief that I could finish in the short time allotted by my military commitment inspired me. I would also like to thank my committee members Dr. Kevin Sowers, Dr. Estelle Russek-Cohen, Dr. Sam Joseph and Dr. Ronald Weiner for their encouragement and helpful suggestions during the course of this research and manuscript preparation. They were always available when I needed them most.

I would like to thank the members of the Colwell laboratory for being kind enough to endure an old student returning to science and for helping me get started in the lab. I wish you all the best of luck in all your endeavors.

I am particularly indebted to the large body of graduate students and post docs who lent their expertise and time teaching me the many techniques I learned during my stay here. These include Chris Grim, Steve Caruso, Joy Watts, Elise Sullivan, Rick Hebert, Cassandra Moe, Erin Lipp, Karin Jackson, Jim Manos, and Liz O'Conner. Without your help I would have spent 100 times the hours reading manuals and troubleshooting the most basic problems.

This work would not have been possible without the additional help from member laboratories at COMB that graciously allowed me to use the equipment that I required. I'm indebted to Dr. Allen Place for his advice on numerous occasions and for lending me everything from chemical standards to GC columns. I was treated as a regular member of the Sowers, Trant, and Schreier labs where I was allowed to work

freely.

Most importantly, I'd like to acknowledge my family for their unfailing love and support. We've endured many missed evenings together, 7-day work weeks, and an obvious lack of quality vacations. To [redacted], I will fulfill my promise to take you to Europe. To [redacted], we'll have more time for finger painting from now on.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

A.	*Acinetobacter*
ANOVA	analysis of variance
aa	amino acids
a_w	water activity
BSA	bovine serum albumin
CMC	critical micelle concentration
DTT	dithiothreitol
FAMES	fatty acid methyl ester
FID	flame ionization detector
GC	gas chromatography
HIC	hydrophobic interaction chromatography
IEF	isoelectric focusing
LNPS	low nitrogen-phosphorous-sulfur medium
LPS	lipopolysaccharide
LSD	least significant difference
P.	*Pseudomonas*
PAGE	polyacrylamide gel electrophoresis
PMSF	phenylmethylsulfonyl fluoride
SE	standard error
WT	wild type

Chapter 1. GENERAL INTRODUCTION

Lipases are glycerol ester hydrolases (EC 3.1.1.3) that catalyze the hydrolysis of triacylglycerols to free fatty acids and glycerol (Fig. 1-1). They resemble esterases in catalytic activity but differ in that their substrates are water-insoluble esters of medium to long-chain fatty acids (Brockerhoff and Jensen 1974). A fundamental difference between the two is the solubility of the substrate in water. Lipases, narrowly defined, are active only against water-insoluble substrates in the form of emulsions, micelles, or aggregates. Lipases have little or no activity toward substrates that are freely soluble because they do not form an interface. In contrast, esterases are active against water-soluble substrates and show no activity toward emulsified substrates.

SEQUENCE HOMOLOGIES AND CLASSIFICATION

Bacterial lipases vary in size, ranging from approximately 20 – 60 kDa and show considerable variation in sequence. They can be classified into eight families, based on conserved sequence motifs, biochemical properties, and mode of secretion (Arpigny and Jaeger 1999). Recently, our laboratory reported the sequence of *lipA,* the putative sequence for an extracellular lipase produced by the commercially important strain, *A. calcoaceticus* RAG-1 (Sullivan 1999). Sequence comparisons between *lipA* and other lipases revealed extensive homologies to Group I lipases (Sullivan *et al.* 1999). These comparisons showed overall homologies greater than

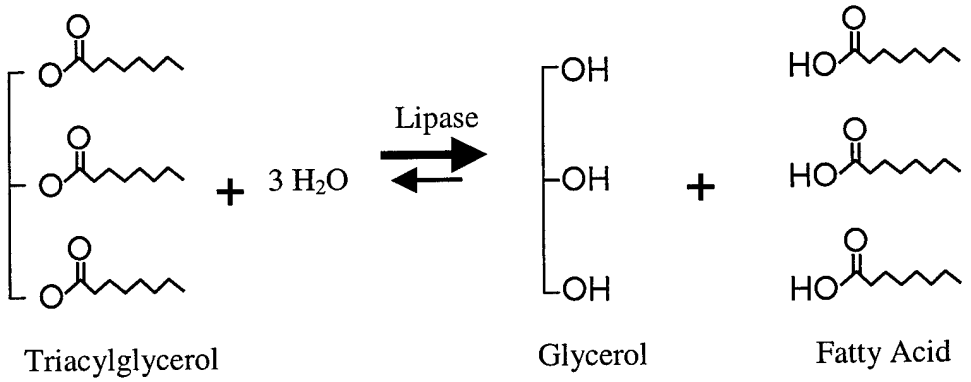


Fig. 1-1. Lypolysis of triacylglerols. Lipases catalyze the reversible hydrolysis of triglycerides to form glycerol and free fatty acids.

49% and were the basis for redefining the group into four subfamilies (Sullivan *et al.* 1999).

Group I Proteobacterial lipases, defined from sequence homologies alone, comprise the largest number of bacterial lipases (Sullivan *et al.* 1999). Its members are derived from the original "*Pseudomonas/Burkholderia*" family described by Gilbert (Gilbert 1993) and include *Pseudomonas* spp., *Proteus vulgaris* K80, *V. cholerae*, *Burkholderia* spp., and *A. calcoaceticus* BD413 (Sullivan *et al.* 1999). Arpigny (Arpigny and Jaeger 1999), using biochemical properties in addition to sequence data, proposed an alternative classification scheme, expanding the group to include lipases from *Chromobacterium viscosum*, *Bacillus* spp., *Staphylococcus* spp., and two strains of *Pseudomonas fluorescens*. Many similarities exist between the two and the differences are mostly indicative of different attempts to consolidate the rapidly growing quantity of new data.

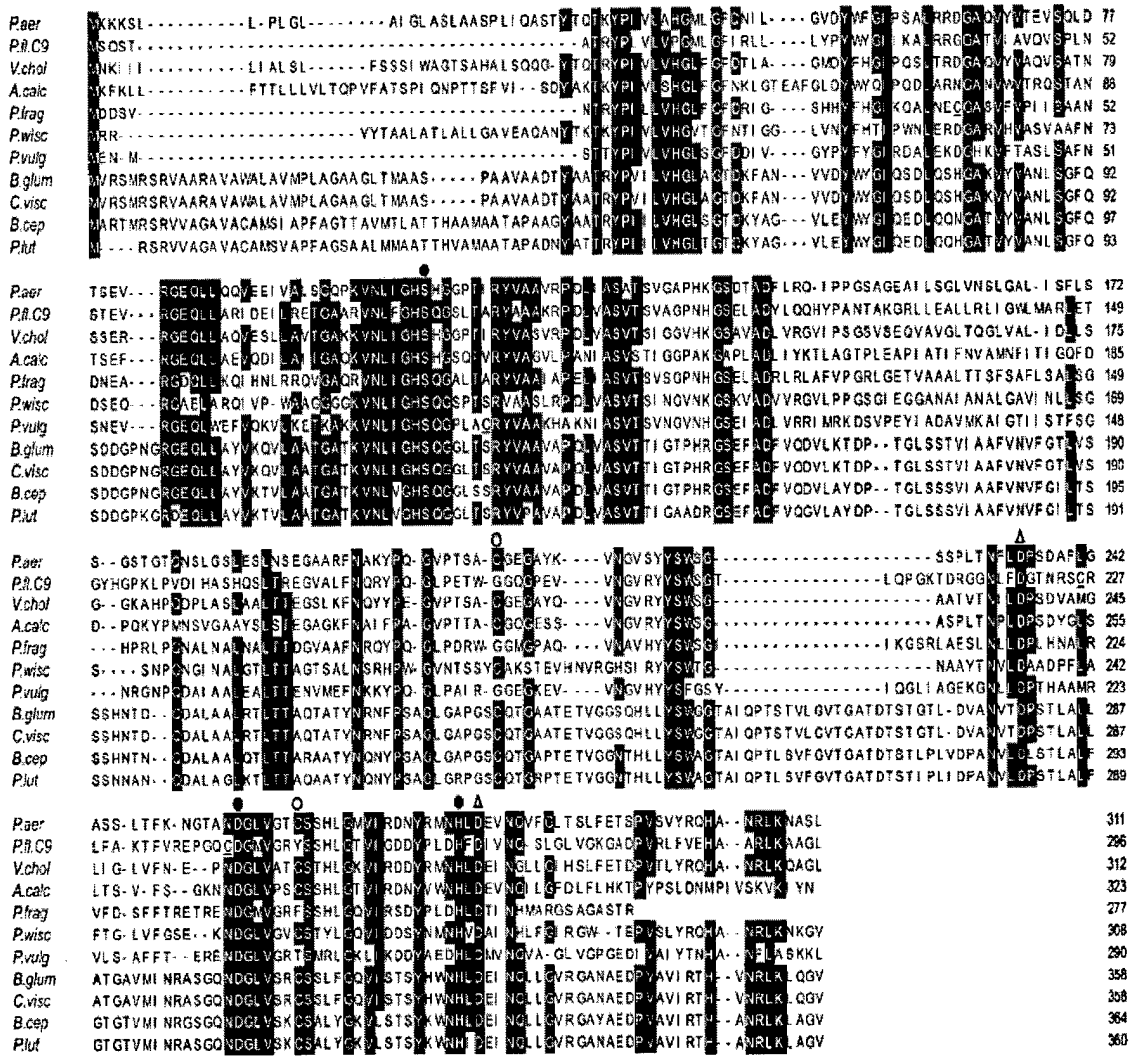


Fig. 1-2. Alignment of amino acid sequences of true lipases from Proteobacterial Group I (Sullivan *et al.* 1999) and subfamilies I.1, and I.2 (Arpigny and Jaeger 1999). Symbols: (•), residues of the catalytic triad; (○) , cysteine residues forming a disulfide bridge; (Δ), aspartic acid residues involved in Ca^{+2}-binding. Adapted from Arpigny and Jaeger, 1999.

Independent of the two classifications are a number of conserved sequences related to catalysis and protein stabilization (Fig. 1-2). The catalytic triad, comprised of Ser, His, and Asp residues, is found at homologous positions in all sequences. Lipases are considered "serine hydrolases" due to the presence of a conserved serine residue that serves as the nucleophilic member in a catalytic triad. Although

structurally distinct, the function of the triad is identical to that of serine proteases, i.e., subtilisin and trypsin. The serine residue is conserved in the pentapeptide Gly-X-Ser-X-Gly, (where X represents any amino acid). The importance of the triad in catalysis has been confirmed by site-directed mutagenesis (Frenken *et al.* 1992) and inhibition studies (Brady *et al.* 1990) in *P.* (formerly *Burkholderia*) *glumae* and *Mucor miehei* lipases, respectively. Further, two Asp residues, identified in crystal structures to be involved in the Ca^{+2} binding site (Lang *et al.* 1996), are also universally conserved. Finally, two cysteine residues forming a single disulfide bridge are conserved in a majority of sequences.

The majority of Group I lipases also share the same mechanism of secretion (type II) using folding catalysts prior to export across the outer membrane (Jaeger *et al.* 1999b; Sullivan *et al.* 1999). Subfamily 1 and 2 (I.1 and I.2, Arpigny and Jaeger 1999) and the *Acinetobacter* clade (Sullivan *et al.* 1999) secrete active lipase dependent upon a chaperone protein named lipase-specific foldase ('Lif' proteins) (Jaeger *et al.* 1994). These proteins are usually encoded in an operon with their cognate lipases where they are putatively transcribed together, although the lipase:Lif ratio is as yet unknown (Jaeger *et al.* 1999b). The Lif proteins are chaperone foldases that are required for proper folding of these lipases in the periplasm (Jaeger *et al.* 1994; Rosenau and Jaeger 2000). Lif proteins from *P. aeruginosa* and *B. glumae* contain a hydrophobic N-terminal sequence that serves to anchor them to the inner membrane and presumably prevents secretion (Frenken *et al.* 1993). The chaperone found in *A. calcoaceticus* BD413, LipB, is anchored in a similar manner. Similarities in sequence identity and production to its counterpart in the pseudomonads has been

reported by Kok (Kok *et al.* 1995b). Disulfide bond formation is thought to take place in the periplasm, mediated by Dsb protein complex (Raina and Missiakas 1997). Finally, active lipase molecules are secreted across the outer membrane by complex protein machinery, the Xcp proteins, consisting of approximately 14 proteins of the general secretion pathway (Pugsley 1993).

BIOCHEMICAL PROPERTIES

Microbial lipases form a remarkably versatile group of enzymes with diverse biochemical properties. Reports describing lipases from *Pseudomonas* spp. are numerous and the enzymes are well characterized. Lipases produced by pseudomonads have drawn considerable attention, probably because they were the first to be studied and have a significant role in industrial applications (Arpigny and Jaeger 1999). However, lipases purified from *Acinetobacter* spp. share many biochemical characteristics with those of *Pseudomonas* spp., indicating significant sequence homologies between the two and possibly lateral gene transfer. Indeed, lipolytic strains of the two genera are often found to coexist in soil, water, and sediment (Blaise and Armstrong 1973; Breuil and Gounot 1972).

Effect of metals

Activity and stability in Group I lipases are often enhanced by cations, with Ca^{+2} being particularly effective (Gilbert *et al.* 1991a). Gilbert (Gilbert *et al.* 1991a) reported significant enhancement in activity due to the presence of $CaCl_2$ in assay mixtures and suggested that the salt reacted with free fatty acids at the interface, increasing surface availability and diminishing charge effects. A similar effect was

reported by Breuil in characterization of a psychrophilic lipase of *Acinetobacter* O_{16} (Breuil and Kushner 1975b). Combined effects of catalytic site activation and increased interface access were suggested as probable mechanisms of Ca^{+2} activation of *B. subtilis* lipase (Lesuisse *et al.* 1993). Stability may also be enhanced by addition of calcium. Bompensieri et al. (Bompensieri *et al.* 1996) reported more than 50% loss in activity by lipase preparations of *Acinetobacter* sp. incubated without $CaCl_2$, while Swaisgood and Bozğlu (Swaisgood and F. Bozoglu 1984) demonstrated a resistance to heat denaturation by *P. fluorescens* MC50 lipase in milk salt buffer.

Inhibition by heavy metals is characteristic of many Group I lipases (Hong and Chang 1988; Ilzumi *et al.* 1990;Yamamoto and N. Fujiwara. 1988). Resistance to inactivation is desirable for many industrial applications (Jaeger and Reetz 1998). Zn^{+2} and Hg^{+2} are inhibitory to many microbial lipases, even at extremely low concentrations ($\leq$ 1 mM), although the exact mechanism of interaction is unclear (Bozoðlu *et al.* 1984; Choo *et al.* 1998; Hong and Chang 1988; Ilzumi *et al.* 1990).

Temperature and pH

Bacterial lipases are stable over wide pH and temperature ranges, but show optimal activity in more narrowly defined ranges. Pseudomonad lipases (reviewed by Gilbert, 1993) may be stable under extremely acidic, e.g., *P. cepacia* (Dünhaupt *et al.* 1991), or alkaline conditions, e.g., *P. cepacia* (Dünhaupt *et al.* 1991) and *P.* fragi (Watanabe *et al.* 1977), but nearly all show pH optima under slightly alkaline conditions (pH 8.0). A psychrophilic lipase prepared from *A. calcoaceticus* LP009 remained active over a wide pH range, with more than 65% of its initial activity being present at pH 4 – 9 (Pratuangdejkul and Dharmsthiti 2000). Bacterial lipases show a

great deal of variation, with respect to temperature tolerance, with many having remarkable stability at high temperatures (Fox and Stepaniak 1983; Ilzumi *et al.* 1990; Kosugi and Kamibayashi 1971). LipP, a cold-adapted lipase purified from *Pseudomonas* sp. B11-1, had maximum activity at 45°C, but the lowest K_m was in the range of 5 to 15°C, consistent with the physiological temperature in the environment from which the bacterium was isolated (Choo *et al.* 1998). *P. aeruginosa* lipase, purified in association with LPS, had remarkable temperature stability (Stuer *et al.* 1986). Cell-free enzyme preparations retained full activity after incubation at room temperature for weeks (Stuer *et al.* 1986). Similar results have been demonstrated using a partially purified lipase from *Acinetobacter* O_{16} associated with phospholipids (Breuil and Kushner 1975b). A word of caution is that comparisons should be made only upon careful consideration of the assay conditions used, because variations in pH and temperature alter the kinetic properties of lipase and also the physiochemical properties of the insoluble substrate (Brockerhoff and Jensen 1974).

Substrate specificity

Lipases are often capable of attacking a wide range of triglycerides and synthetic substrates of varying acyl chain lengths, but may show specificity toward substrates with medium ($C_6 - C_8$) or long ($\geq C_{10}$) fatty acid chains. Lipases from *A. calcoaceticus* BD413 (Kok *et al.* 1995a) and *A. radioresistens* CMC-1 (Hong and Chang 1988) hydrolyze esters of *p*-nitrophenyl (*p*NP) of chain lengths from C_8 to C_{18}, but maximum activity was demonstrated against *p*-nitrophenyl palmitate (C_{16}). In contrast, *Acinetobacter* sp. OPA 55 (Markweg-Hanke *et al.* 1995) and *A.*

calcoaceticus LP009 (Pratuangdejkul and Dharmsthiti 2000) produce lipases that preferentially attack substrates containing medium chain fatty acid moieties, determined by ethyl ester and monoacid triglycerides hydrolysis, respectively. Similar substrate specificity (C_8 and C_{10}) was shown for *Pseudomonas* sp. (ATCC 2108) lipase hydrolyzing saturated fats, although a significant increase in activity occurred if long chain triglycerides contained unsaturated fatty acids (Kordel *et al.* 1991). Substrate specificity is a critical factor in selecting lipases for modification of fats and oils and for the production of structured triglycerides or 'nutraceutical' agents (Björkling *et al.* 1991; Jaeger and Reetz 1998).

Interfacial Activation

Biochemical evidence for interfacial activation was first established by measuring activity of pancreatic lipase at different substrate concentrations above and below saturation. Sarda and Desneulle (Desnuelle 1961; Sarda and P. Desnuelle 1958) found little lipolytic activity at substrate concentrations below saturation (where the substrates exist in monomeric form), but demonstrated a rapid increase in activity at higher concentrations, upon formation of emulsions. In comparison, horse liver esterase followed Michaelis-Menton kinetics, with maximum reaction rate attained prior to substrate saturation. The esterase was active only on substrate monomers, reaching V_{max} prior to emulsion formation (Sarda and P. Desnuelle 1958).

Because activity is dependent on the presence of an interface, lipolysis does not follow strict Michaelis-Menten kinetics, where the enzyme and substrate are completely soluble in one homogeneous phase. Adsorption imparts a dimensional

change to any kinetic model of lipolysis, where the quantity of enzyme is best described as a function of the surface area (moles/unit surface area) rather than molar concentration (moles/unit volume). New kinetic models have been derived for lipases that account for both adsorption and the formation of the enzyme-substrate complex (Verger 1976). The model assumes that adsorption is reversible and water-soluble enzyme is in equilibrium with lipase fixed at the interface. Activation is often concomitant with adsorption leading to a favorable energy state for the enzyme (Verger 1976). The enzyme-substrate complex follows a two-dimensional Michaelis-Menten scheme, with instantaneous solubilization of the reaction products assumed (Ransac *et al.* 1999). Equations have been derived that perfectly fit the experimental data (Ransac *et al.* 1999; Verger 1976).

Kinetic and biochemical studies of interfaces have revealed their importance in lipolysis. Reaction rates may be affected by access, available surface area, and physiochemistry. Free fatty acids and/or partial glycerides may accumulate at the interface during lipolysis and have been shown to block enzyme binding (1983). Conversely, addition of salts to the reaction medium may cause free fatty acids to precipitate, increasing activity by enhancing enzyme adsorption (Iwai *et al.* 1970; Liu *et al.* 1973). Verger *et al.* (Gargouri *et al.* 1984; Verger *et al.* 1991) reported inhibition of lipase adsorption by proteins, e.g., bovine serum albumin, which they interpreted to be desorption of the lipase from the substrate because of a change in the interfacial quality. Surfactants also have an effect upon lipase adsorption and activity. In general, concentrations below the critical micelle concentration (CMC) increase enzyme activity and higher concentrations are inhibitory. However, this

interpretation is an oversimplification since surface-active compounds actually have a complex combination of effects on lipase, substrate, and interface.

The chemical nature of the interface also has an effect on enzyme binding. Several investigators (Tsujita and Brockman 1987; Tsujita *et al.* 1989; Tsujita *et al.* 1987) have found that adsorption of pancreatic carboxyl-ester lipase to fatty acid or acyl glycerol monolayer films lacked chemical specificity, but showed strong correlation to pH and ionization state. In addition, binding to mixed films containing phospholipids and 1, 3-diolein was reduced to 5 – 10% of the values for pure diolein films. Activity is also dependent upon available substrate surface area. Smaller emulsified droplets provide greater area/volume ratio than larger micelles (Benzonana and P. Desnuelle 1965).

THREE-DIMENSIONAL STRUCTURE OF LIPASES

The three-dimensional structure of a bacterial lipase was constructed only after the structure of several mammalian and fungal lipase enzymes had been established. The first bacterial lipase to be crystallized came from *P.* (*Burkholderia*) *glumae* (PGL) (Noble *et al.* 1993). Subsequently, lipases from *C. viscosum* (CVL) (Lang *et al.* 1996) and *B. cepacia* (PCL) (Kim *et al.* 1997) were determined at greater resolution. Nardini *et al.* (Nardini *et al.* 2000) were the first to determine the three-dimensional structure of a Group I.1 lipase (*P. aeruginosa,* PAL) in the "open" configuration. Bacterial lipase structure has gained increasing attention because these enzymes are widely used in biotransformations and various stereo-selective reactions. Three-dimensional structures provide a basis for understanding biochemical and

enantio-selective characteristics, in addition to insight into the potential of tailoring lipases for selected applications.

Catalytic domain

Despite sequence variations, all bacterial lipases presumably share the same general structure. This belief is based upon the three-dimensional structures published to date that place bacterial lipases in the α/β hydrolase superfamily, whose members include a diverse range of hydrolytic enzymes (Ollis *et al.* 1992). The "prototypic" catalytic domain of this hydrolase superfamily contains eight β-strands alternating with six α-helices (Nardini *et al.* 2000; Noble *et al.* 1993; Ollis *et al.* 1992). The nucleophilic serine is always the central residue in an extremely sharp turn between strand five and helix C, forming a strand—nucleophile—helix structure, called the nucleophilic elbow (Ollis *et al.* 1992). The nucleophilic elbow is the most highly conserved feature of the α/β hydrolase family and allows the serine to project into the active site, allowing interaction with the catalytic histidine and substrate (Fig. 1-3). Steric hindrance is possible between opposing side chains of the strand and helix elbow but is avoided by limiting interfering residues to those with short chains that are, in most cases glycine (R=H), resulting in the highly conserved Gly-X-Ser-X-Gly pentapeptide (Ollis *et al.* 1992).

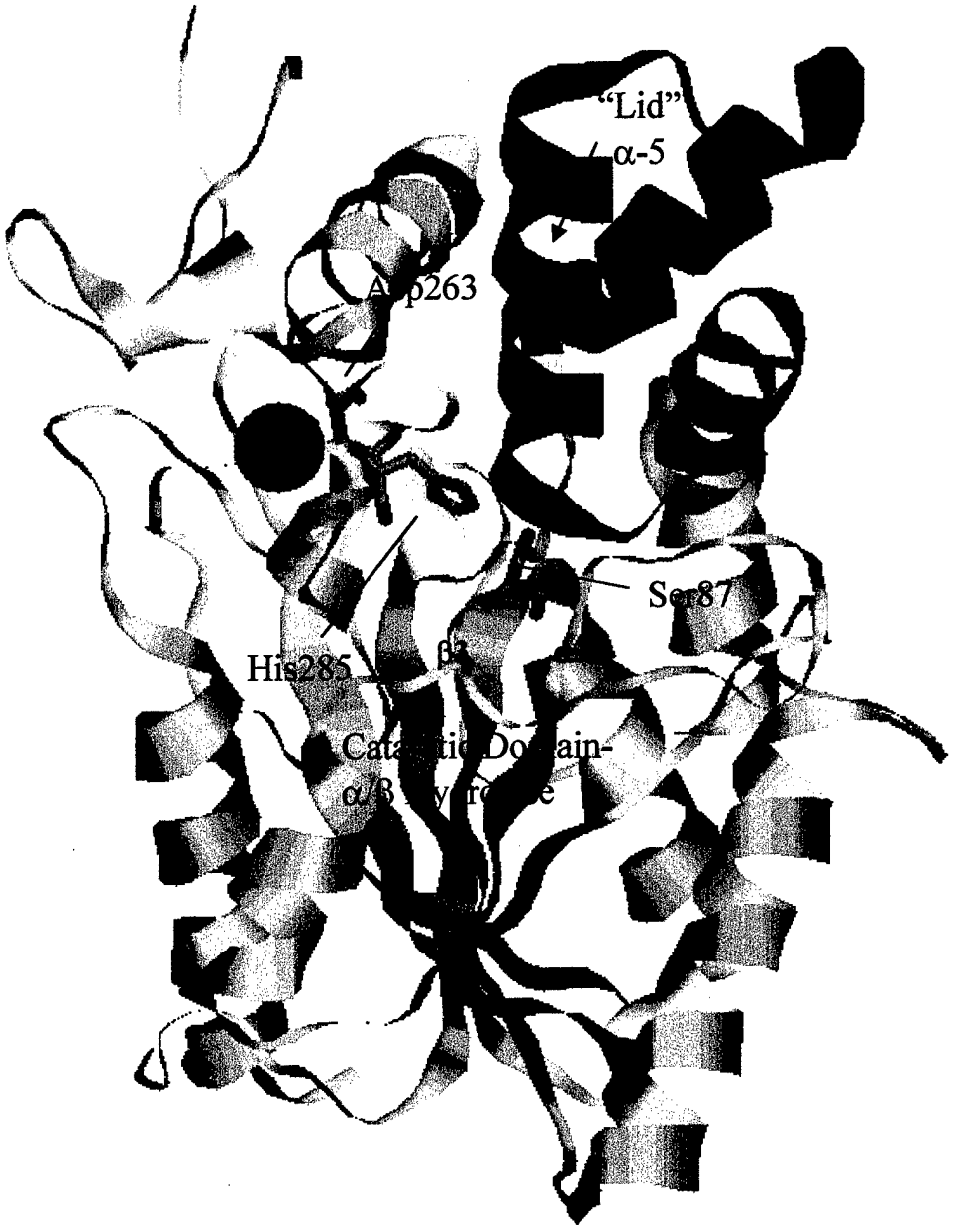


Fig. 1-3. Three-dimensional structure of *P.* (*Burkholderia*) *glumae* lipase. The three domains are indicated by color. Residues of the catalytic triad are depicted as "sticks" in the CPK color scheme (red, oxygen; blue, nitrogen). The central nucleophile between β3 and α3 forms the nucleophilic elbow (β3 and α3 are equivalent to β5 and αC, respectively, in the nomenclature of Ollis *et al.*, 1992). Drawn with RASMOL (Sayle and Milner-White 1995).

All bacterial lipase structures that have been resolved to date contain the canonical α/β fold, with minor variations. PGL, CVL, and PCL contain only six

parallel β strands in the central β sheet (Kim *et al.* 1997; Lang *et al.* 1996; Noble *et al.* 1993). The C-terminal antiparallel β sheet in PAL is missing, resulting is a more compact molecule (Nardini *et al.* 2000). Greater variations can be expected for smaller lipases, such as that of *B. subtilis* (Ransac *et al.* 1994).

Active site

Crystallographic data available for both eukaryotic and bacterial lipases reveal active sites deeply recessed within the molecules (Brady *et al.* 1990; Noble *et al.* 1993; Winkler *et al.* 1990). The residues surrounding the catalytic site are predominantly hydrophobic, forming a deep pocket containing active-site residues (Kim *et al.* 1997). Comparison of lipases in the "closed" (Noble *et al.* 1993), partially open (Lang *et al.* 1996), and "open" configurations suggest only the open configuration allows solvent access to the catalytic site (Kim *et al.* 1997). Schrag et al. (Schrag *et al.* 1997) demonstrated a cleft leading to active site residues is divided into two channels. Further, when crystallized in complex with triglyceride analogs, *B. cepacia* (PCL) lipase revealed the active site to consist of a large, hydrophobic groove bound to the *sn*-3 fatty acid and another channel strongly binding the *sn*-2 chain through hydrophilic and hydrophobic interactions, while the *sn*-1 fatty acid is only weakly bound (Lang and Dijkstra 1998). Enantio-selectivity in PCL is predominantly determined by the size and interactions of the *sn*-2 chain, and size of the *sn*-3 chain (Lang and Dijkstra 1998). Similar results were obtained for PCL crystallized with other inhibitors (Lang *et al.* 1998).

Ca^{+2}-binding site

Other conserved features provide stability to the molecule. Group I bacterial lipases contain a bound Ca^{+2} in close proximity to the catalytic site (Lang *et al.* 1996; Nardini *et al.* 2000; Noble *et al.* 1993). Comparative structural data show two Asp residues are highly conserved members of the calcium-binding site. Sequence comparisons of *lipA* from *A. calcoaceticus* RAG-1 with other Group I lipases predicts Asp^{240} and Asp^{282} to be putative Ca^{+2}-binding residues (Sullivan 1999). The Ca^{+2} ion in crystallized lipase prepared from *Pseudomonas aeruginosa* (PAL) shows octahedral coordination (Nardini *et al.* 2000) and its loss (with accompanying disruption of h-bonding network) influences main and side-chain conformations in the loop containing the active-site histidine, resulting in enzyme inactivation (Fig. 1-4) (Lang *et al.* 1996). A single disulfide linkage is highly conserved in bacterial lipases and is believed to provide structural support to the molecule (Jaeger *et al.* 1999a; Nardini *et al.* 2000).

Structural basis of interfacial activation

Lipolytic enzymes are characterized by increased activity at the lipid-water interface of micellar substrates, a phenomenon termed interfacial activation (Sarda and P. Desnuelle 1958). Crystallized enzymes, complexed with transition state analogs, have provided a basis for understanding the structural rearrangements involving this phenomenon (Brzozowski *et al.* 1991; van Tilbeurgh *et al.* 1993).

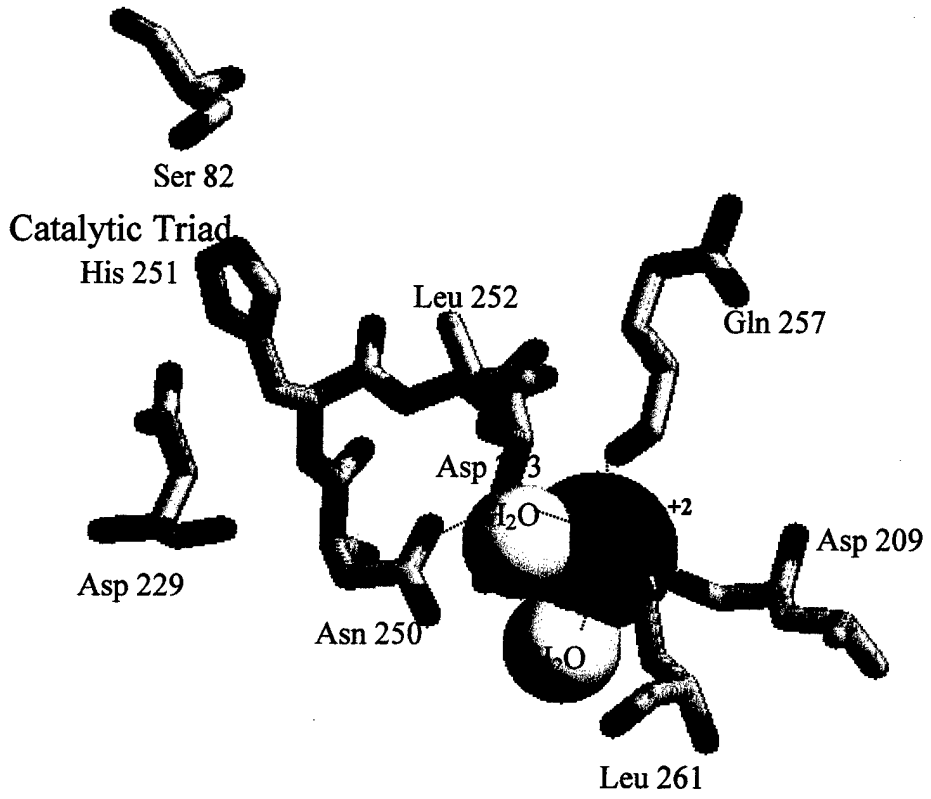


Fig. 1-4. Ca^{+2} binding site of PAL. The Ca^{+2} ion shows octahedral coordination (dashed lines) with Asp^{209} and Asp^{253}, Gln^{257}, and Leu^{261}, and two water molecules. The distance between Ca^{+2} and the nucleophile Ser^{82} is approximately 15Å. Loss of the Ca^{+2} ion results in a concomitant change in the hydrogen-bonding network that leads to active site His^{285} and deactivation. Adapted from Nardini *et al.*, 2000. Drawn using RASMOL (Sayle and Milner-White 1995).

In the absence of a lipid-water interface, solvent access to the catalytic triad is blocked, making the catalytic site inaccessible to the substrate. Two domains cover the site, one of which acts as a movable hinge or "lid" during activation (below), while the other domain also extends into the bulk solvent (Noble *et al.* 1993). Since the catalytic center is recessed deep within the protein, the conformation suggests limited catalytic efficiency. This seemingly ineffective design is overcome by a

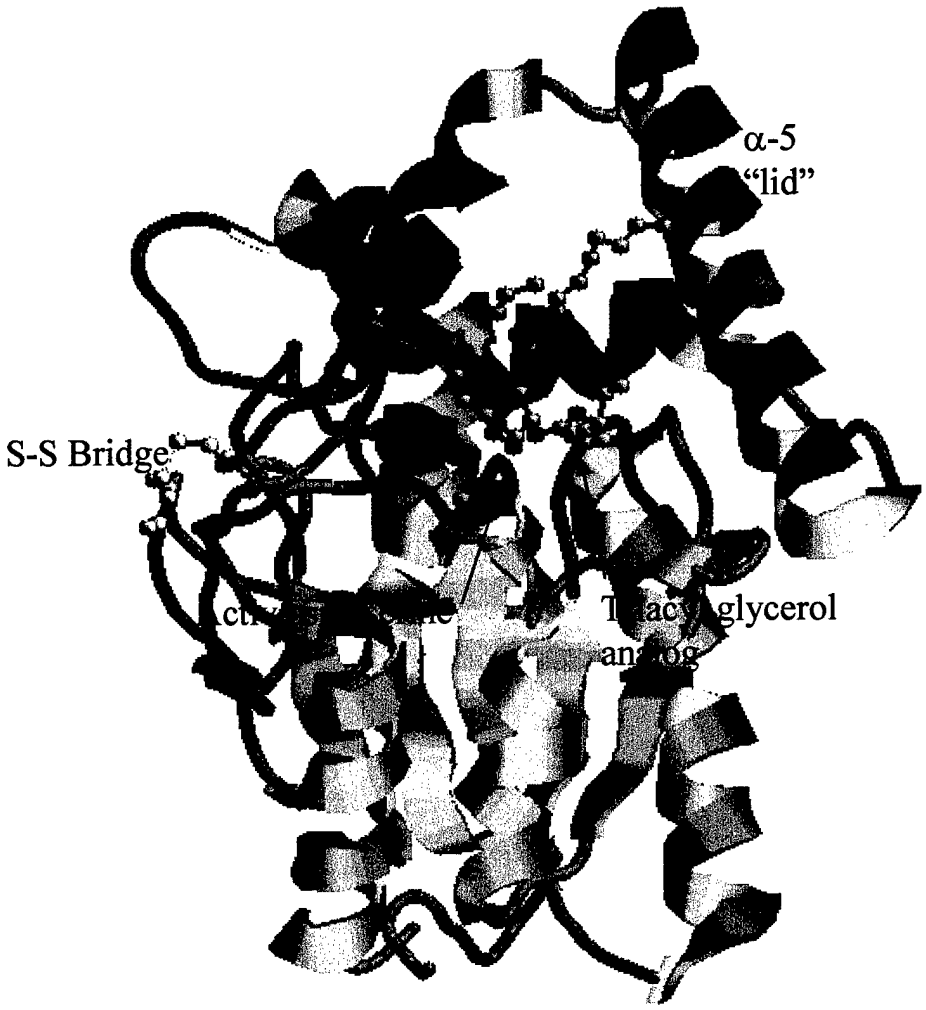


Fig. 1-5. *P. aeruginosa* lipase in the open conformation. Hydrophobic portions of the α-helices of the cap domain involved in substrate binding, along with hydrophobic residues surrounding the catalytic pocket, are shown in blue. Residues forming the disulfide bridge are shown in yellow. The triacylglycerol analog (inhibitor) is covalently bound to Ser^{82} and depicted in ball and stick representation. Drawn by RASMOL (Sayle and Milner-White 1995).

movable α-helix or "lid" which opens upon contact with the interface. During activation, the lid rotates away from the molecule, exposing hydrophobic residues toward the substrate and improving contact with the binding domain. This structural change provides access to the hydrophobic interior and active site (Fig. 1-5). In

addition, when the lid opens, residues are placed that form an oxyanion hole in a position to stabilize the tetrahedral intermediate that is formed during catalysis (Kim *et al.* 1997). The oxyanion hole has been demonstrated in PAL crystallized in open conformation (Kim *et al.* 1997), and CVL partially open (Lang *et al.* 1996). It is absent in the closed structure of PGL (Noble *et al.* 1993).

It must be noted that all lipases do not demonstrate interfacial activation. Examples include the 19-kDa lipases from *B. subtilis* (Lesuisse *et al.* 1993) and PAL from *P. aeruginosa* (Jaeger *et al.* 1993). Lipase from *B. subtilis* lacks a lid covering access to the active site in the absence of lipid-water interfaces (Ransac *et al.* 1994). The explanation is less clear for PAL because the presence of a lid has been clearly demonstrated (Nardini *et al.* 2000). There are a number of exceptions of enzymes possessing a lid but not exhibiting interfacial activation. Thus, lipases can be more simply defined as carboxylesterases hydrolyzing long chain acytriglycerols (Ferrato *et al.* 1997).

BIOTECHNOLOGICAL APPLICATION OF LIPASES

In 1998, sales of enzymes worldwide were estimated to be $1 billion (Rao *et al.* 1998), representing nearly 70% increase above figures first reported in 1989, indicating a rapid expansion of the industry (Arbige and W.H. Pitcher 1989). Sales of lipase products have grown in step with the industry, but represent only 3% of total enzyme sales (Rao *et al.* 1998). This sum is impressive, considering that the detergent industry alone is responsible for approximately 1,000 tons of lipases sold per year (Godfrey and West 1996).

Lipases are selected for industrial applications based on their unique biochemical properties. The food industry uses lipases for flavoring cheese with specific fatty acids (Stead 1986), synthesizing cocoa butter substitute from inexpensive oils (Harwood 1989; Jaeger and Reetz 1998), flavoring milk products, and processing fats and oils (Yamamoto and N. Fujiwara. 1988). More recently, fungal lipases from *Rhizomucor miehei* (Jennings and C. C. Akoh 2000) and *Rhizopus japonicus* (Mogi *et al.* 2000) are being investigated for their potential in producing structured triglycerides that may be able to alleviate fat absorption abnormalities in patients suffering from cystic fibrosis, colitis, and pancreatic deficiencies. In the detergent industry, lipases with broad substrate specificity, high pH tolerance, and compatibility with chelating and oxidizing agents are used in conjunction with other enzyme products (protease, amylase, and cellulase) (Rao *et al.* 1998). Lipolase (Novo Industri A/S), a product derived from *Humicola lanuginose,* was first introduced in 1988 and is used by the two largest detergent manufacturers, Procter and Gamble and Unilever. Lumafast (Genencor, USA) and Lipomax (Gist-brocades, Netherlands), two products derived from *Pseudomonas* spp., are also commonly found in commercial detergent formulations (Jaeger *et al.* 1994).

Organic synthesis

Lipolysis (Fig. 1-1) in aqueous solution is reversible, but thermodynamically unfavorable. However, it is possible to perform synthesis reactions in water-limiting systems, making feasible the production of many compounds previously thought unattainable, except through chemical processes. Reactions can take place under

nearly dry solvent conditions and may have the advantage of higher activity, enhanced thermostability (Zaks and Klibanov 1984), and stereo-selectivity (Kirchner and M. P. Scollar 1985). The number of investigations describing properties of lipases in such systems is increasing dramatically (Jaeger *et al.* 1999b). Lipases have proven to be versatile enzymes in organic solvent systems that catalyze the synthesis of various esters, triglycerides, and optically pure compounds. Products currently available were developed for application in resolution of racemic alcohols and acids, asymmetric synthesis, and esterifaction/transesterifaction reactions (Amano Enzyme Inc. 2000). Kinetic resolution of racemates commonly results in conversions near the theoretical limit (≅ 50%) and enantiomeric excess (% ee) exceeding 90% (Amano Enzyme Inc. 2000).

Lipases show regio- and stereo-selectivity during catalytic conversions, making them ideal candidates for the production of enantiomer pure compounds used in the pharmaceutical and agricultural industries. Lipase from *Chromobacterium viscosum* was shown to prefer saturated long-chain fatty acid methyl esters in transesterifaction with 1-propanol and to acylate sorbitol in a regioselective manner (Barros *et al.* 1994). Irreversible enantio- and regio-selective syntheses of glycerol derivatives, sugars, and alcohols have been achieved, using lipases from *Pseudomonas*, *Candida cylindracea*, and porcine pancreas, employing enol esters as acylating agents (Wang *et al.* 1988). In addition, directed acylation of sucrose to produce emulsifiers, sweeteners, and surfactants is possible by employing lipases from *Humicola* and *Candida* as catalysts (Ferrer *et al.* 1999).

NATURAL ROLE OF LIPASE PRODUCTION

Lipases as virulence factors

Interest in lipases has increased recently due to the recognition of lipases as important virulence factors. Their role in pathogenesis has not been specifically defined but they are generally believed to work in combination with other extracellular enzymes responsible for colonization and membrane damage (Jaeger *et al.* 1994). *Propionibacterium acnes* releases an extracellular lipase that breaks down sebum triglycerides of sebaceous glands, producing free fatty acids which may predispose hosts to acne (Ingham *et al.* 1981). The fatty acids may also promote cell-to-cell adherence within the follicles, implicating the lipase in an indirect role as a colonization factor (Gribbon *et al.* 1993). Patients suffering from cystic fibrosis are often infected with *P. aeruginosa*, a cause of lung infection and still considered the main cause of death (Doring 1993). Studies suggest two lipolytic enzymes (lipase and phopholipase) work synergistically, degrading dipalmitoylphophatidyl-choline, the major lung surfactant, and promoting inflammatory reponse from human platlets (Konig *et al.* 1994).

The role of extracellular lipases found in other clinical strains is less clear. A lipase from another potentially pathogenic strain found on skin, *S. epidermidis*, has been cloned, sequenced, and purified, but its role in pathogenesis has not been determined (Farrell *et al.* 1993). *S. aureus* produces a lipase, when residing in infected patients, that may block human granulocyte function (Christensson *et al.* 1985; Rollof *et al.* 1988). Anti-lipase IgG antibodies have been demonstrated, indicating active lipase secretion (Christensson *et al.* 1985).

Hydrocarbon degradation

There is a correlation between the ability to utilize hydrocarbons and lipase production (Schindler *et al.* 1975). The effect of crude oil on bacterial populations revealed strains that could metabolize hydrocarbons produced lipase, whereas strains that showed no lipolytic activity could not utilize hexadecane. Breuil et al. (Breuil *et al.* 1978) observed a similar result for *Acinetobacter lwoffi* strain O_{16} and *P. aeruginosa*, but also noted that production of lipase by other bacteria included in their study did not always guarantee utilization of hydrocarbons. *A. lwoffi* strain O_{16}, isolated from polluted river sediment, showed lipase induction when grown on alkanes but the investigators were unable to determine a role in hydrocarbon degradation (Breuil and Kushner 1975a; Breuil *et al.* 1975; Breuil *et al.* 1978).

Lipase production during growth on alkanes remains puzzling because hydrocarbons and their degradation intermediates are not lipase substrates. The most common pathway by which alkanes are metabolized by microorganisms is via monoterminal oxidation ($RCH_3 \rightarrow RCH_2OH \rightarrow RCHO \rightarrow RCOOH$), as described for *P. oleovorans* (van Beilen *et al.* 1994) and *Acinetobacter* sp. ADP-1 (Geissdorfer *et al.* 1999). This pathway is catalyzed by a three-component monooxygenase, composed of alkane hydroxylase, rubredoxin, and rubredoxin reductase (Geissdorfer *et al.* 1999). An alternative pathway has been postulated for some *Acinetobacter* spp. (Finnerty 1988). In this pathway, a dioxygenase creates a hydroperoxide intermediate that is also converted to free fatty acids. Evidence for the existence of the dioxygenase has been presented for *Acinetobacter* sp. strains M-1 and RAG-1 (Maeng *et al.* 1996). The production of free fatty acids is the termination step in both

pathways. Fatty acids may be further catabolized by β-oxidation or incorporated into lipids. Lipase has not been shown to play any direct role in alkane utilization. However, it has been postulated that fatty acids produced by alkane metabolism or triglyceride hydrolysis may aid uptake of alkanes through emulsification of the substrate and lipid membrane substitution (Breuil and Kushner 1980)

HISTORICAL BACKGROUND OF *ACINETOBACTER* SP. RAG-1 AND ITS EMULSAN

Emulsan Production by *Acinetobacter* sp. RAG-1

Acinetobacter sp. RAG-1 was isolated from seawater by crude oil enrichment as part of a bacterial consortium capable of hydrocarbon degradation and dispersion (Reisfeld *et al.* 1972). The strain was originally studied for its ability to degrade hydrocarbons but is now more noted for its production of a powerful emulsifying agent, termed emulsan (Gutnick *et al.* 1991; Rosenberg *et al.* 1979). Emulsan is produced as a polymeric minicapsule that is released into the growth medium at the end of exponential growth or during periods of starvation where it is an active emulsifier (Rosenberg *et al.* 1979; Goldman *et al.* 1982). As one of the most powerful emulsion stabilizers known today, it is effective at concentrations as low as 0.01 – 0.001% (Desai and Banat 1997). Emulsan demonstrates significant substrate specificity—it does not emulsify pure aliphatics, aromatics or cyclic hydrocarbons effectively, but shows greater activity toward mixtures of aliphatic and/or cyclic alkanes (Rosenberg *et al.* 1979; Zosim *et al.* 1982). Emulsan shows maximal activity

at pH 5.0 – 7.5 but its activity above pH 6.0 is dependent on presence of divalent cations (Rosenberg *et al.* 1979).

Emulsan structure

Emulsan is a large anionic heteropolysaccharide with an average molecular weight of 9.9×10^5 (Zuckerberg *et al.* 1979). Its major components consist of a repeating trisaccharide unit of *N*-acetyl-D-galactosamine, *N*-acetylgalactosamine uronic acid, and diamino-6-deoxy-D-glucose, with covalently attached fatty acids (Fig.1-6) (Belsky *et al.* 1979; Gorkovenko *et al.* 1997). Carboxyl groups of the uronic acid contribute to the hydrophilic nature of the backbone and a pK_a of 3.05 (Zuckerberg *et al.* 1979). Fatty acids are attached to the sugar component by *N*-acyl and *O*-ester linkages (Zuckerberg *et al.* 1979). Their composition varies with growth conditions but total dry weight does not exceed 5 - 15% of the polymer (Belsky *et al.* 1979). Acyl chain lengths vary, normally between $C_{10} - C_{18}$, with 2-hydroxy and 3-hydroxydodecanonoic predominant (Belsky *et al.* 1979; Gorkovenko *et al.* 1997). Non-covalently bound protein is associated with emulsan and may comprise up to 15% of total dry weight. The protein moiety is not required for emulsification but does enhance activity up to 60% (Zuckerberg *et al.* 1979). In addition, the protein component is reported to be non-specific, with bovine serim album (BSA), hexokinase, and lysozyme acting as suitable replacements (Zuckerberg *et al.* 1979).

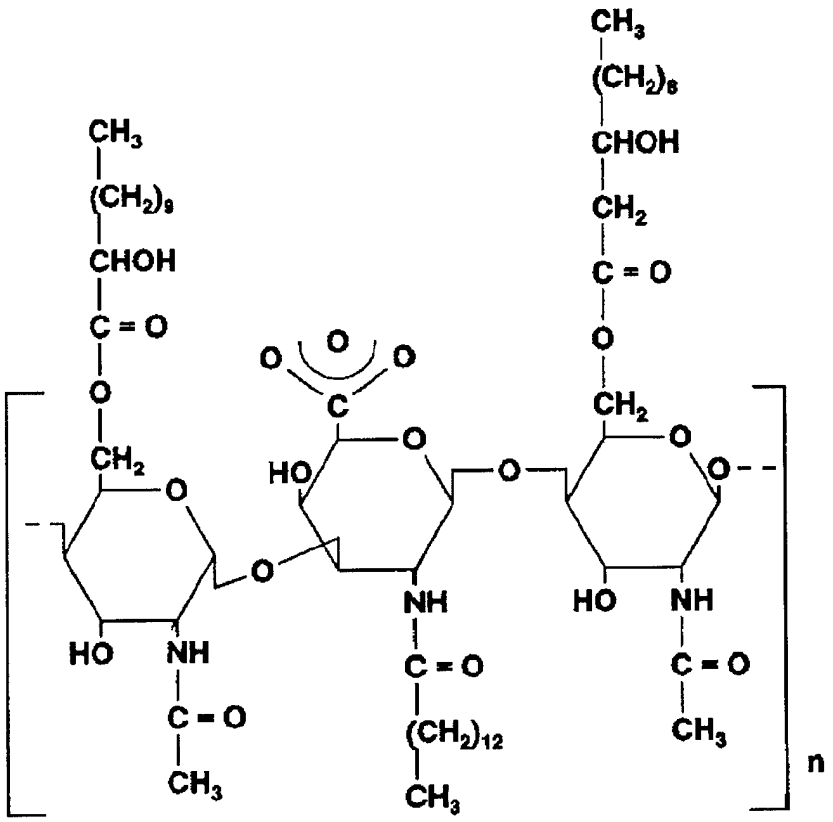


Fig. 1-6. Structure of emulsan produced by *Acinetobacter* sp. RAG-1. (Desai and Banat 1997).

Conformation changes of emulsan in solution

The combination of anionic polysaccharide and hydrophobic fatty acids give emulsan its unique amphipathic properties. Cell free emulsan is an emulsion stabilizer; it does not work by creating emulsions. In oil and water mixtures, it exists either in free solution or at the oil-water interface where it colonizes the oil droplet, forming a thin film. Its large size and negative charge (due to carboxyl groups) prevents droplet coalescence. Based on phage binding studies, it has been suggested by Pines and Gutnick (Gutnick 1987; Pines and Gutnick 1984) that conformation of

emulsan is the same at the cell surface and in the oil-water interface, but is relaxed (open) in free solution. Upon association with the protein component, viscosity is lowered, indicating a conformational change in the emulsan to a less extended form (Zosim *et al.* 1987). At the oil-water interface, the conformation allows fatty acids to contact the oil droplet and polysaccharide exposed to the bulk solvent (Gutnick 1987). This changes the surface properties of the oil droplet by making it hydrophilic.

Role of emulsan in hydrocarbon degradation

Pines and Gutnick (Pines and Gutnick 1986) observed that cell-bound emulsan was required for growth of RAG-1 on hydrocarbons. The observation that emulsan was a cell surface polymer (Goldman *et al.* 1982; Pines *et al.* 1983) suggested it may be involved with adhesion to hydrocarbons (Rosenberg *et al.* 1989). However, synthesis of emulsan by cells attached to oil droplets renders them more hydrophilic and leads to desorption from the interface (Rosenberg *et al.* 1983b). Moreover, treatment of human buccal epithelial cells with emulsan results in a 70% reduction in the adherent autochthonous bacterial flora (Rosenberg *et al.* 1983a).

Emulsan during growth of bacteria on hydrocarbons is best described as a desorption factor. Hydrophobic RAG-1 cells attach to oil droplets by means of thin fimbriae (Rosenberg *et al.* 1982). During the growth phase, cells form a biofilm at the interface and accumulate emulsan on their surface in the form of a capsule (Rosenberg *et al.* 1989). Emulsan acts to reduce interfacial tension between hydrocarbon and water, allowing increased hydrocarbon uptake and emulsion

formation (Baldi *et al.* 1999). RAG-1 cells remain attached to the oil droplets until all utilizable alkanes are depleted. The capsule is released into the growth medium in an esterase-mediated process (Shabtai and Gutnick 1985) where by, the polymer attaches to oil-water interfaces, inhibiting cell reattachment to "marked" droplets. Cells, free of the capsule, are able to colonize new droplets. This hypothesis is supported by the fact that the capsule is released during stationary phase and periods of starvation and reduced protein synthesis (Gutnick 1987; Rubinovitz *et al.* 1982). These data suggest that *Acinetobacter* may use surfactant production to regulate its cell surface properties, dependent upon physiological requirements (Rosenberg 1993).

Correlation of lipase production and emulsifying activity

Previous research in our laboratory showed a positive correlation between emulsification and lipase activities by RAG-1 cells grown on hexadecane (Leahy 1993). Further, a linear relationship was demonstrated between lipase activity and fatty acid content per mole of emulsan and emulsification production (Leahy 1993). The increase in lipase activity that was observed was commensurate with increased specific fatty acid content of the polymer. These data provided the basis of the hypothesis that RAG-1 lipase directly modifies the fatty acid content of emulsan through initial esterification or post-synthesis transesterification reactions (Leahy 1993). Evidence supporting this view was several fold: 1) $LipA^-$ cells were found to have lower specific fatty acid content and emulsifying activity than wild type (WT) cells, 2) emulsans from a complement mutant strain ($LipA^+$, a lipase "overproducer") contained greater fatty acid content and emulsifying activity than WT cells, and 3)

lipase activity showed both positive and negative correlation with hexadecenoic acid and an unidentified fatty acid, respectively. Further, other investigators reported that cleavage of fatty acids from the emulsan decreased emulsifying activity by more than 50% (Rosenberg *et al.* 1979).

In this study, two separate analyses of comparative fatty acid composition of emulsans were performed, yielding mixed results. Leahy (Leahy 1993) demonstrated both qualitative and quantitative differences in emulsan fatty acids from LipA$^-$ and WT cells grown in hexadecane, while Sullivan (Sullivan 1999) could not detect a significant difference between the two. The lack of agreement between these studies can be explained by either a lack of quantitative GC standards, improved harvest procedures used in the later study, or different growth phases at the time the cells were harvested (stationary vs. late logarithmic phase, respectively). Further, synthesis of emulsan has been shown to be a dynamic and adaptive process that is dependent upon the growth and substrate conditions employed (Gorkovenko *et al.* 1997; Zhang *et al.* 1997).

OBJECTIVES OF THIS STUDY

In this study, we investigated the role of extracellular lipase in RAG-1 cells grown on hexadecane and triglycerides (lipase substrates). In contrast to previous studies, an *in vitro* approach was employed, using purified lipase, to determine if enzymatic modification of emulsans by lipase-catalyzed transesterification is a natural, widely occurring phenomenon. The biochemical properties of purified LipA

were extensively investigated and its potential value for various industrial processes assessed.

One objective was to understand the spatial and temporal distribution of lipase production during growth on hexadecane and triglycerides. To accomplish this, lipase activity of whole culture, cell-bound, and cell-free fractions were determined separately. Previous investigations excluded one or more of these fractions. Based on whole culture sampling, it had been reported that lipase production paralleled growth; i.e., was not a growth-phase-dependent phenomenon. This hypothesis was further tested because of its contradiction with respect to results obtained for other *Acinetobacter* spp. (Bompensieri *et al.* 1996; Fischer *et al.* 1987; Kok *et al.* 1996) and in comparison with growth on triglycerides.

For *in vitro* transesterification experiments, a separation method was established to obtain pure lipase preparations. Lipases have been purified by many different techniques, but application of hydrophobic interaction chromatography (HIC) to purify dilute extracellular proteins is particularly well suited in this case because of the high-load capacity of the gel, capability in binding proteins by direct incubation with the gel matrix, and easy scalability (Scopes 1994). The method employed in this study was first to solubilize lipase with detergent, followed by cell-free concentration, ion exchange, and HIC.

The second objective of the study was to establish a biochemical characterization of the pure lipase that would serve two purposes. First, by understanding the properties of the purified enzyme, an optimum method to investigate lipase-catalyzed transesterification of emulsan could be developed. That

is, data describing substrate specificity, the effects of pH, temperature, and organic solvent on enzyme activity would elucidate conditions under which the transesterifaction of the polymer was possible. Second, lipases have had significant attention drawn to them because of their ability to catalyze a variety of reactions in non-aqueous media (Jaeger and Reetz 1998). Moreover, there is a rapidly growing requirement for specialized enzymes in various industrial processes. Enzyme characterization allows a general assessment of LipA for potential use in the rapidly growing field of industrial catalyst applications.

The final objectives of this study were to: (1) determine if, and under which conditions, lipase may change the fatty acid structure of emulsan, and (2) determine the characteristics of any analogs that could be created. A continuing goal of studies on emulsans, as with other naturally occurring biosurfactants, has been to identify new and innovative uses for these powerful emulsifying agents (Rosenberg and Ron 1999). In addition, production of surfactant analogs of natural surfactants is the subject of much attention, reviewed by Desai and Banat (Desai and Banat 1997). More specifically, Gorkovenko (Gorkovenko *et al.* 1995; Gorkovenko *et al.* 1997; Zhang *et al.* 1997) demonstrated that changes in emulsan fatty acid chemistry are possible by substituting various fatty acids as carbon sources in the growth medium. Despite the many investigations cited, only limited improvement in emulsification properties of emulsan analogs that have been created has been achieved (Zhang *et al.* 1997). By taking advantage of the stereo- and regio-specificity of lipase catalysis, it may be possible to develop a novel approach to the creation of emulsan analogs, thereby permitting a more direct method for their creation.

Chapter 2. PURIFICATION OF RAG-1 LIPASE

CHAPTER ABSTRACT

Spatial and temporal production of an extracellular lipase, LipA, by *Acinetobacter* sp. RAG-1 cells grown in a minimal medium supplemented with hexadecane, trilaurin, or tripalmitin as sole carbon source was examined, with the intent of determining a suitable medium for protein purification. LipA, produced by cells grown in hexadecane, showed high activity and stability; hence this medium was selected for the purification studies. LipA was purified from concentrated supernatants of cells grown to stationary phase, when the majority of the enzyme is released. RAG-1 lipase was purified, with a 22% yield and greater than 10-fold purification. The protein showed little affinity for the anion exchange resins employed in this study and contaminating supernatant proteins were removed by passing crude supernatants over a Mono Q column. The lipase was bound to a butyl sepharose column and eluted with increasing Triton X-100 concentration. The native protein was purified as a high molecular weight aggregate, determined by gel filtration chromatography. A single major protein band with an apparent molecular weight of 33 kDa was observed in denaturing SDS PAGE gels. The lipase remained highly active after native gel electrophoresis and demonstrated hydrolytic activity toward emulsions of both medium and long chain triglycerides, determined by activity staining (zymogram).

INTRODUCTION

Lipases (EC 3.1.1.3) are triacyl glycerol hydrolases that catalyze the breakdown and synthesis of esters of long-chain fatty acids and glycerol. In aqueous systems, hydrolysis reactions are favored, i.e., the breakdown of tri-, di-, and monoglycerides into free fatty acids and glycerol, while in systems with low water content, the synthesis of esters may predominate. Recently, interest in microbial lipases has received increased attention because of the discovery that they remain enzymatically active in organic solvents, thereby making them ideal tools as biocatalysts in a variety of organic synthesis reactions and biotransformations, of which many have industrial applications. Additionally, interest in bacterial lipases has stemmed from their identification as important virulence factors (Jaeger *et al.* 1994). Properties of lipases that lend great potential to biotechnological applications include high enantioselectivity, broad substrate specificity, and the lack of a cofactor requirement (Jaeger and Reetz 1998). In addition, the enzymes are easily recyclable as lyophilized powders or when immobilized on supports, prolonging their utility in this form.

Many microbial lipases are available as commercial products. Fungal lipases from *Candida*, *Thermomyces* (*Humicola*), *Aspergillus*, and *Rhizomucor* spp. are used in organic synthesis, food processing, and as detergent additives and bacterial lipases from *Pseudomonas*, *Burkholderia*, and *Chromobacterium* spp. are used in similar applications (Jaeger and Reetz 1998; Amano Enzyme Inc. 2000). Among bacterial lipases, those from *Pseudomonas* spp. predominate in industrial applications,

probably due to the fact that they were among the first bacterial lipases to be purified, characterized, and studied extensively (Arpigny and Jaeger 1999).

Although lipolytic strains of *Acinetobacter* are well documented (Breuil *et al.* 1975; Blaise and Armstrong 1973; Walker *et al.* 1975), only a small number of these lipases have been purified and their properties examined. The lack of literature regarding *Acinetobacter* lipases does not seem justified, as they are closely related to those of *Pseudomonas* spp. in sequence (Sullivan *et al.* 1999; Kok *et al.* 1995a) and biochemistry (Arpigny and Jaeger 1999). The biochemical properties of purified *Acinetobacter* lipases are summarized in Table 2-1. Jaeger (Jaeger *et al.* 1999b; Arpigny and Jaeger 1999), using sequence comparisons of LipA from *A. calcoaceticus* BD413 (Accession no. X80800) (Kok *et al.* 1995a) to family "type" lipase from *P. aeruginosa,* classified *Acinetobacter* lipases as true lipases, family **I**.1. Other lipases in this family include those from *Pseudomonas* spp., *Vibrio cholerae*, and *Proteus vulgaris*, all of which reportedly possess molecular masses in the range 30 – 32 kDa (Arpigny and Jaeger 1999). However, as the data in Table 2-1 suggest, lipases of *Acinetobacter* species are more variable, with respect to molecular mass, than other lipases in family **I**.1.

Recently, we reported on the cloning and sequencing of an extracellular lipase (LipA) from *Acinetobacter* sp. RAG-1, which contains several conserved regions common to bacterial lipases (Sullivan *et al.* 1999). This strain also has been characterized with respect to its production of emulsan, a powerful emulsifying agent (Gutnick *et al.* 1991; Gutnick *et al.* 1991; Zuckerberg *et al.* 1979).

Table 2-1. Biochemical properties of *Acinetobacter* lipases.

Name	MW (k Da)	Remarks	Reference
Acinetobacter O_{16}	≥ 200	purified as high MW aggregate, temp. optimum 35°C, pH optimum 8.5, stimulated by Ca^{+2}	Breuil and Kusner 1975b
Acinetobacter radioresistens CMC-1	45	alkaline lipase, optimum temp 40°C, pH optimum 10.5, inhibited by Zn^{+2} and PMSF	Hong and Chang 1988
A. calcoaceticus AAC323-1	NR[1]	stabilized by Ca^{+2}	Bompensieri *et al.* 1996
A. calcoaceticus BD413	32.1	specificity toward LCFA[2], pH optimum 7.8 – 8.8	Kok *et al.* 1995a
A. calcoaceticus LP009	23	temp optimum 50°C, pH optimum 7.0, inhibited by EDTA	Pratuangdejkul and Dharmsthiti 2000
Acinetobacter nov. sp. strain KM109	62	Temp optimum 45°C, pH optimum 8.0, affinity for benzoyl esters	Mitsuhashi *et al.* 1999

[1] NR = not reported, strain a high lipase producing derivative of BD413, M_r assumed to be 32 kDa
[2] LCFA = long chain fatty acids

Based on deduced aa sequence of LipA and comparison with corresponding sequences from other bacterial lipases, we derived a classification scheme similar to that of Jaeger (Jaeger *et al.* 1999b) and presented a more detailed analysis of family I.1 (Sullivan *et al.* 1999). The phylogenetic tree we developed showed an "*Acinetobacter*" clade within the family and further predicted low sequence homologies (50 % aa identity) within the clade, indicating significant divergence. See Table 2-1.

In the study reported here, the production of an extracellular lipase from *Acinetobacter* sp. RAG-1 was demonstrated during growth on minimal media amended with hexadecane, or fat (tripalmitin or trilaurin), as sole carbon source. In addition, we succeeded in purifying RAG-1 lipase produced in hexadecane-grown

cells, demonstrating its activity toward medium and long chain acyl glycerols. The hydrolytic properties of LipA suggest it belongs in the family of true lipases, Family I. (Arpigny and Jaeger 1999).

MATERIALS AND METHODS

Media and culture conditions

Cells of *Acinetobacter* sp. RAG-1 (ATCC 31012) recovered from frozen stock (-80°C) were used to inoculate Spirit Blue agar containing lipase reagent (Difco, Liverpool, Australia) and incubated overnight at 37°C. Single colonies were selected for inoculating into 100 ml of a low nitrogen, phosphorous, sulfur (LNPS) medium consisting of (per liter): KH_2PO_4, 3.3g; Na_2HPO_4, 2.2g; Na_2SO_4, 1.0g; NH_4NO_3, 1.0g; NaCl, 5.0g; $MgSO_4$, 0.29g; $CaCl_2$, 0.05g; and $FeSO_4$, 1 mg (pH 7.0). Tripalmitin, trilaurin, or hexadecane (10 mM) was employed as sole carbon source. Inocula were grown to mid-exponential phase at 30°C and 200 rpm in a rotary incubator/shaker (New Brunswick Scientific, Edison, NJ). Aliquots of cultures were transferred to fresh LNPS (1:30 dilution) supplemented with the appropriate carbon source for growth and lipase production.

Lipase Assay

Lipase activity was measured as hydrolysis of *p*-nitrophenyl palmitate (*p*NPP) in deoxycholate buffer, as described by Fischer (Fischer *et al.* 1987). Samples (20 µl – 500 µl) were added to pre-warmed (30°C) phosphate buffer (50 mM, pH 8) containing 0.2% sodium deoxycholate and 0.1% gum arabic, final volume 3.0 ml.

The mixture was incubated for 5 min at 30°C. *p*NPP (0.30 mM final concentration) was added and the mixture shaken, allowing the reaction to proceed for 3 min. Lipase activity was determined by the rate of *p*-nitrophenol production, measured at 405 nm in a model DU640 spectrophotometer (Beckman Coulter, Fullerton, CA). Lipolytic activity was determined, using substrate free blanks as control. The reaction rate was calculated from the slope of the absorbance curve, using software installed by the manufacturer (Beckman Coulter). The extinction coefficient under the conditions described was 17,454 L mol·cm^{-1} (Leahy 1993). One unit of enzyme activity is defined as the amount of enzyme forming 1 μmol of *p*NP min^{-1} ml^{-1}.

During growth studies, total lipase activity was determined from aliquots of whole cultures, as previously described (Sullivan 1999). Cell bound and cell free activities were determined separately, as follows: cells were pelleted (10,000 rpm, 5 minutes), washed twice; and resuspended in sterile LNPS. Supernatants were filtered (0.2μm, Tuffryn membrane, Gelman Labs) prior to assay.

Measurement of protein concentration

Protein concentration was measured by the method of Bradford (Bradford 1976), using bovine serum albumin (BSA) as the standard. Where appropriate, the detergent-compatible BCA protein assay (Pierce, Rockford, Il) was used to determine protein concentration in samples containing Triton X-100. Total protein of the cellular fractions was determined after cell disruption using **a** Branson model 450 (Branson Instruments, Danbury, CT) sonicator fitted with a 1 mm microtip. Protein

concentration was routinely used as a measure of cell growth in hydrophobic media (Marin *et al.* 1995; Zhang and Miller 1994).

Emulsifying activity

Emulsifying activity was determined as previously described (Rosenberg *et al.* 1979) in 125 ml Erlenmeyer flasks containing 0.1 – 1.0 ml emulsifying agent (5 – 75 μg), 0.1 ml of 1:1 (v/v) hexadecane and 2-methylnaphthalene, and 20 mM Tris-HCl buffer (pH 7.2) containing 10 mM $MgSO_4$ to a final volume of 7.5 ml. Flasks were capped and shaken for 1 hr using a Burnell wrist action shaker (55% duty cycle). Contents of the flasks were transferred to Klett tubes and turbidity measurements made using a Klett-Summerson colorimeter (Klett Mfg., New York) fitted with a green filter and employing distilled water blanks. Dilutions were made using distilled water to adjust readings to 30 – 150 Klett units. All measurements were corrected for turbidity caused by hydrocarbon emulsion alone. Emulsan concentrations were determined (as appropriate) from a standard curve prepared in an identical manner, using purified, deproteinated emulsan (Petroferm Fernandina Beach, FL).

Lipase Purification

Results of preliminary growth studies demonstrated that RAG-1 lipase had an enhanced stability in minimal medium amended with hexadecane, compared to triglyceride, as a carbon source. For this reason, LNPS amended with 10 mM hexadecane was selected as the purification medium. After incubation for 48 hr in LNPS amended with 10mM hexadecane, cells were removed by centrifugation

(10,000 x g) at 4°C and the supernatants pooled. To increase the yield of lipase, 2 mM $CaCl_2$ and 2 mM $MgCl_2$ were added during magnetic stirring and the crude supernatant was centrifuged a second time. To remove residual hexadecane, the combined sample was allowed to stand for 30 min prior to passage through a course glass fiber filter, after which the sample was filtered (0.2 μm).

Supernatants were concentrated by ultrafiltration, using an Amicon RA2000 filter unit fitted with an S1Y10 spiral membrane (10000MW cut-off, 20 psi) and the sample volume was reduced approximately 10-15 fold to 200 ml. After concentration, a serine protease inhibitor, phenylmethylsulfonyl fluoride (PMSF), was added (0.2 mM) to reduce loss from proteolytic activity. Concentrated supernatants were ultracentrifuged at 28,000 rpm (141,000 x g) for 1 hr at 4 °C. Supernatants containing lipase were divided into 35 ml aliquots and stored at -80°C prior to chromatography.

Preliminary experiments to investigate binding properties of the lipase, using anion (Mono Q) and cation (Mono S) exchange resins, showed little (10%) affinity for the matrices under the conditions employed. However, other proteins were effectively bound, resulting in significant purification. Therefore, anion exchange was employed as a preliminary step for hydrophobic interaction chromatography (HIC). Samples were dialyzed overnight against 20 mM TRIS-HCl buffer (pH 8.0), followed by passage through two Econo-Pac Mono Q cartridges (Bio-Rad, Hercules, CA) fitted in tandem at approximately 1 ml min^{-1}, prior to loading on the hydrophobic matrix.

HIC methods were employed, as described elsewhere (Bompensieri *et al.* 1996; Kok *et al.* 1995a). An equal volume of Buffer 1 (30 mM TRIS-HCl; 2 mM $CaCl_2$; 2 mM $MgCl_2$; 0.5 M NaCl) was added to supernatant that had equilibrated at room temperature for 30 min. Aliquots were added to 25 ml of Butyl Sepharose Fast Flow 4 hydrophobic medium (Amersham Pharmacia Biotech, NJ) equilibrated in the same buffer. Equilibrating the medium and samples at room temperature allowed for more than 90% of the lipase to be bound. The protein slurry was degassed and loaded onto a glass Econo-Column (Bio-Rad) fitted with a column adaptor, for a total column volume of 20 – 25 ml. Lipase was eluted using two linear gradient profiles and flow rate of 1 ml min^{-1}. Two column volumes of Buffer 1 were passed through the column, followed by two volumes of a decreasing salt gradient (0.5M – 0M NaCl in Buffer 1) and washed in six volumes of the same buffer without salt. A gradient of 10 column volumes of Triton X-100 (0 – 1.0%) in 30 mM TRIS-HCl pH 8.0, followed by an additional 60 ml 1% Triton X-100, was used to elute LipA and other highly hydrophobic proteins. Absorbance (280 nm) was monitored and fractions (8.0 ml) collected from the detergent gradient were assayed for lipase activity. Fractions containing pure lipase, based on SDS PAGE results, were pooled and concentrated, using ultrafiltration (PM10 membrane, Millipore, Bedford, MA) or an Ultrafree-4 centrifugal filter unit (10000 MW cut-off, Millipore) and stored at –80°C.

Detergent removal

Triton X-100 was removed from the protein preparations by incubating samples overnight at 4 °C with the polymeric absorbent, Amberlite XAD-2 (Sigma, St. Louis,

MO). The absorbent was cleaned following the manufacturer's instructions and fine particles removed by siphoning after each of several washes in distilled water. The absorbent was equilibrated in 30 mM TRIS-HCl pH 8.0 prior to use. Under these conditions, approximately 50 mg detergent was removed per gram (wet) of absorbent. The absorbent was removed from the supernatant by centrifugation (4000 rpm, 4 °C).

Triton X-100 concentration (%, v/v) in protein samples was determined against a standard curve prepared in the following manner. Preliminary ultraviolet scan of Triton X-100 and protein (bovine serum albumin, BSA) revealed that each demonstrated absorbance throughout the ultraviolet range. The difference ($Abs_{(triton)} - Abs_{(protein)}$) was calculated for each wavelength and plotted against wavelength. The wavelength at which the greatest difference in absorbance was observed (289 nm) was selected for preparation of a standard curve, using Triton X-100 concentrations between 0 – 1.0% and found to be linear between 0 – 0.3% detergent.

Gel Electrophoresis

SDS-PAGE gel electrophoresis was performed according to the methods of Laemmli (Laemmli 1970). Protein samples were dissolved in 2X sample buffer containing (per 8.0 ml): distilled water, 4.9 ml; 0.5M Tris-HCl pH 6.8, 1.0 ml; 10% (w/v) SDS, 1.6 ml; β-mercaptoethanol, 0.4 ml; 1% (w/v) bromophenol blue, 0.1 ml; and urea, 7.6g. Gels were cast at 4% (stacking), and 15% (separation) with the addition of 5 M urea and 0.1% (w/v) SDS. Gels were prepared from stock solution containing acrylamide:bisacrylamide in the ratio of 37.5:1. Stacking gels were buffered with 0.125 M Tris-HCl pH 6.8, while the separation gel consisted of 0.375

M Tris-HCl pH 8.8. Samples of mixed proteins (crude supernatant, ion exchange) contained 50μg protein per lane while highly purified samples contained 10μg protein per lane. Electrophoresis was performed in the cold (4°C) using 100 mV for stacking and 200 mV for separation. Marker proteins (Bio Rad) were used for molecular weight determinations (in daltons): myosin (200,000), β-galactosidase (116,250), phosphorylase b (97,400), serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), trypsin inhibitor (21,500), lysozyme (14,400), and aprotinin (6,500). Proteins were stained with Coomassie Blue R-250 stain, consisting of (per liter): methanol, 400 ml; acetic acid, 100 ml; Coomassie blue R-250, 1 g; water, 500 ml. Destaining solution consisted of 50% methanol in deionized water.

Non-denaturing or native PAGE was performed using the discontinuous gel system of Orstein and Davis (Ornstein 1964; Davis ;Davis). Protein samples (10-15 μg) were dissolved in 0.0625 M Tris-HCl pH 6.8 buffer with 0.025% (v/v) glycerol. Gels were cast with a 4 % stacking and 6 % resolving gel with 0.125 M Tris-HCl pH 6.8 and 0.375 M Tris-HCl pH 8.8 buffers, respectively. Electrophoresis was performed at 4°C and proteins were allowed to stack at 80mV for approximately 30 min and separate at 160mV for 1 hr. Prior to incubation with zymograms, native gels were rinsed three times with distilled water and equilibrated in 30 mM TRIS-HCl pH 8.0 containing 1% Triton X-100 for 30 min at 25°C.

Isoelectric focusing (IEF) determination of LipA pI was performed using precast IEF gels, according to manufacturer's instructions (Bio-Rad). The anode and cathode buffers consisted of 7 mM phosphoric acid and 20 mM lysine/20 mM arginine, respectively. IEF standards were purchased from Sigma and consisted of

trypsin inhibitor (pI 4.6); carbonic anhydrase (pI 6.6); and lentil lectin (pI 8.2, 8.6, 8.8). Protein samples and standards (10 μg) in 25% glycerol were centrifuged for 2 min at 4°C. Gel wells were rinsed with cathode buffer and 5 μl of 15% glycerol was used to coat the wells. Samples were loaded and run using a three tier, constant-volt protocol consisting of 100 volts, 1 hr; 250 volts, 1 hr; and 500 volts, 30 min. IEF gels were fixed for 30 min in 4% (v/v) sulfosalicylic acid, 12.5%(w/v) trichloracetic acid, and 30% (v/v) methanol. Gels were rinsed in three changes of 7.5% (v/v) acetic acid for 1 hr and further equilibrated in 0.05% (w/v) SDS and 7.5% acetic acid. Gels were allowed to stand overnight in 7.5% acetic acid prior to staining. Gels were stained in a 1:5000 dilution of sypro orange stain (Molecular Probes, Eugene, OR) in 7.5% acetic acid for 30 min prior to visualizing on the fluorimager.

Gel filtration (size exclusion) chromatography

Protein samples were concentrated (15 – 20 fold) by ultrafiltration using Ultrafree-4 centrifugal filter units (10000 MW cut-off, Millipore). Samples (125 µl) were loaded via syringe onto 50 cm x 1 cm (ID) glass column packed with approximately 40 ml Bio-Gel P-60 (Bio-Rad) matrix (fractionation range, 3 – 60 kDa) equilibrated in elution buffer (30mM TRIS HCl/0.01% Triton X-100, pH 8.0) and fitted with a flow adapter (Bio-Rad). Samples were eluted at 4°C at a flow rate of 3.9 ml hr^{-1} (5 cm hr^{-1} linear rate) and monitored for protein content at 280 nm. Fractions (1.0 ml) were collected and assayed for lipase activity under standard conditions. Molecular weight determinations of LipA were obtained by comparison

to a standard curve prepared from gel filtration standards (Sigma) eluted from the column under identical conditions.

Activity Staining (Zymogram)

Zymogram staining was accomplished by two methods. LipA activity against triolein (olive oil) was demonstrated according to the method of Gilbert (Gilbert *et al.* 1991a). In summary, gel overlays were prepared from a 5% olive oil (Sigma) emulsion in 50 mM TRIS-HCl (pH 8.5) containing 0.01% (w/v) Victoria Blue B dye (pH indicator) and 1.3% agarose (Fisher Scientific, Pittsburgh, PA). Victoria Blue B was added as a solution in 70% ethanol. Zymograms were also prepared from an emulsion of 1% tricaprylin ($C_{8:0}$) in 25 mM TRIS-HCl and 5 mM $CaCl_2$ (pH 8.0) (Sommer *et al.* 1997). Emulsions were created by the method of Yadav et al. (Yadav *et al.* 1998), by addition of 1.5% (w/v) gum to the reagents blended at high speed for 3 min. Gels were cast between glass plates and allowed to stand at 4°C for 5 min. Activity staining was accomplished by overlaying native gels with the zymograms in a closed glass dish and incubating at 37°C from 4 to 16 hr. The incubation chamber was kept humid by addition of paper toweling saturated with TRIS buffer. Lipase activity against olive oil was recorded by appearance of a dark blue band. Positive indicator of tricaprylin hydrolysis was recorded by appearance of a zone of clearing.

RESULTS AND DISCUSSION

Selection of purification medium

Acinetobacter sp. RAG-1 cells were grown in a minimal medium (LNPS) amended with 10 mM hexadecane, trilaurin, or tripalmitin as sole carbon and energy source and assayed for lipase production in order to determine the most suitable medium for purification. High volumetric lipase activity had been previously reported in RAG-1 cells on media containing the nonlipase substrate hexadecane but the stability of the enzyme in this medium was not investigated (Leahy 1993). More nutrient rich media (LB, nutrient broth) were not screened for lipase production due to possible interference of proteinaceous contaminants in rich media (Kok *et al.* 1995a).

RAG-1 cells grown in the presence of hexadecane produced high levels of extracellular lipase that was growth phase dependent (Fig. 2-1). Under these conditions, only a small amount of lipase activity was detected during exponential growth, but a 10-fold increase in activity occurred during early stationary phase. This result suggests hexadecane does not induce lipase production, but rather the alkane or one of its degradation products represses *lipA* expression. This may not be surprising as alkanes are non-lipase substrates and a *lipA*$^-$ strain (Sullivan 1999) is able to utilize hexadecane (Fig. 2-1). Kok (Kok *et al.* 1996) reported repression of *lipA* in *A. calcoaceticus* AAC321-1 (measured by β-galactosidase expression in the *lipA*::*lacZ* strain) grown in hexadecane and attributed it to the production of fatty acids (hexadecanoic acid, an intermediate of hexadecane utilization) during catabolism of the alkane. Gilbert (Gilbert *et al.* 1991b) measured free oleic acid concentration in

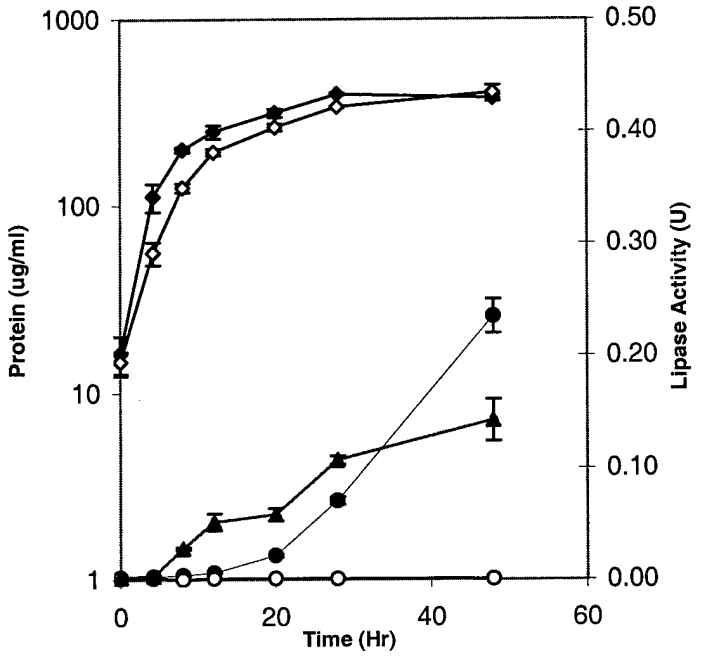


Fig. 2-1. Growth and lipase production by *Acinetobacter* sp. RAG-1 in LNPS medium supplemented with 10 mM hexadecane. Growth (diamonds) of RAG-1 WT (closed symbols) and LipA$^-$ strain (open symbols) is measured as total protein (μg ml^{-1}). Cell-free (circles) and cell bound (▲) lipase activities were determined under standard conditions. One unit of activity is defined as the amount of enzyme that released 1μmole of *p*-nitrophenol min^{-1} ml^{-1} under the assay conditions used. Data are presented as means of at least three replicates.

culture supernatants of *Pseudomonas aeruginosa* EF2 grown on Tween 80 (polyoxyethylene sorbitan oleyl ester) as sole carbon source and demonstrated a significant decrease in free fatty acids that was correlated with a several fold increase in lipase activity at cessation of exponential growth. She attributed the increase in lipase activity to relief in fatty acid repression and weak induction by carbon limitation. The data presented here are in good agreement with these studies andsuggest fatty acid repression may be a conserved method for regulation in family I.1 lipases.

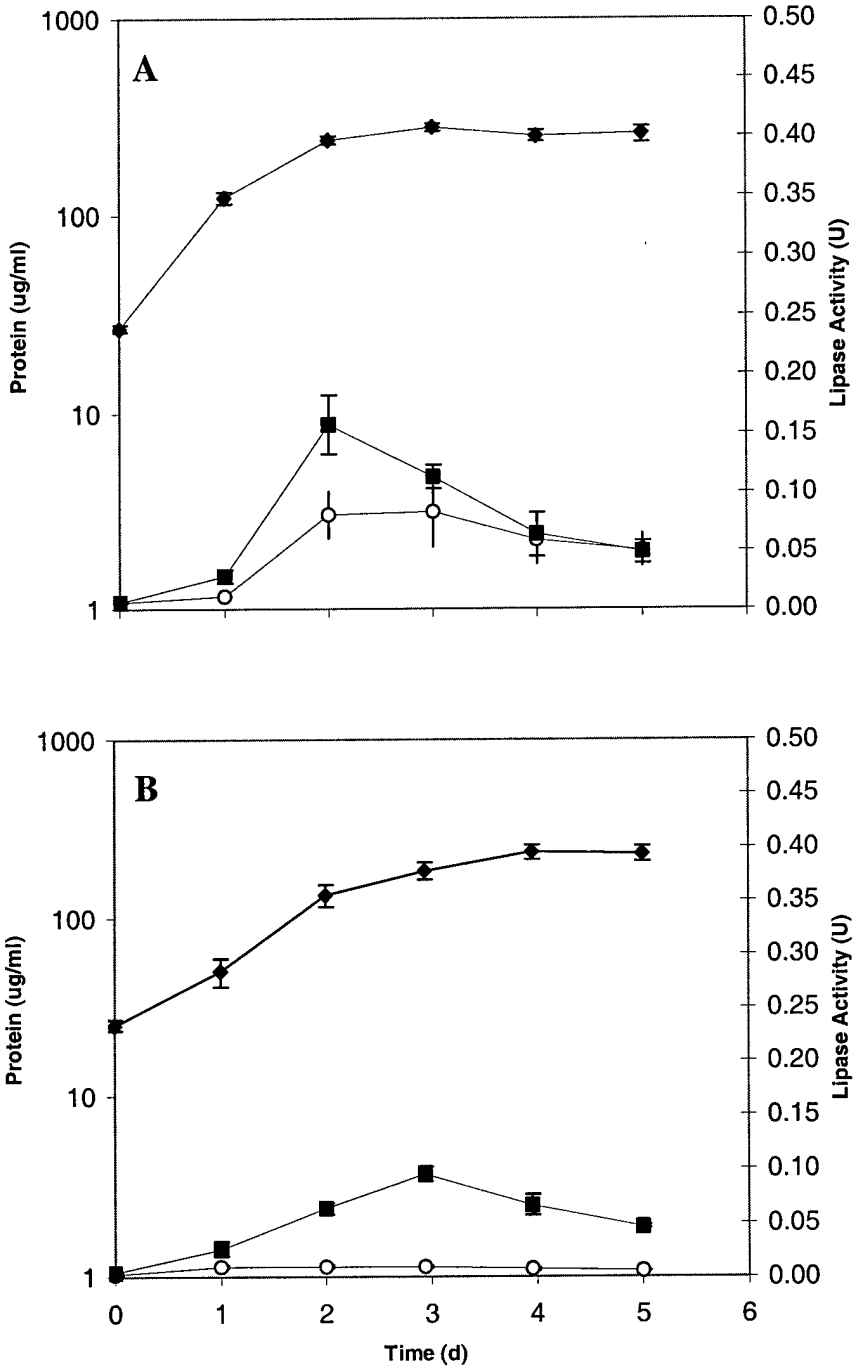


Fig. 2-2. Growth and lipase production by *Acinetobacter* sp. RAG-1 during growth in LNPS medium supplemented with 10 mM trilaurin, (A) and tripalmitin, (B) as sole carbon source. Growth (protein concentration (μg/ml), (♦); cell-bound activity, (■); cell-free activity (o). Data presented are means of at least three replicates. One unit of activity is defined as the amount of enzyme that releases 1μmole of p-nitrophenol min^{-1} ml^{-1} under the assay conditions used.

Growth and lipase production experiments using trilaurin and tripalmitin as carbon source are more difficult to interpret. Growth in LNPS plus triglyceride media was slow, reaching stationary phase in two (trilaurin) to three (tripalmitin) days (Fig. 2-2). This is probably due to the limited solubility of these carbon sources at 30°C. Additionally, little cell free lipase activity was detected in the growth medium even though studies with a *lipA*$^-$ strain showed the enzyme is required for growth in the presence of triglycerides (not shown). Lipase levels produced in triglyceride media were low compared with that produced in similar studies utilizing hexadecane.

Early in exponential growth, a small increase in cell-bound lipase activity was detected, suggesting that triglycerides are weak inducers of *lipA* (Fig. 2-2). As growth ceased, a sharp increase in cell-bound activity occurred. The increase may be a result of cessation of fatty acid repression as reported in growth studies using hexadecane (Kok *et al.* 1993). It is of interest to note that even after cell-bound activity increased, cell-free lipase activity remained very low. Further, as stationary phase progressed, cell-bound activity decreased sharply, indicating that the lipase may be regulated by some other mechanism, such as proteolysis. Regulation by proteolytic degradation has been proposed by others to explain activity loss during growth on triolein (Kok *et al.* 1996) and sorbitan monoleate (Gilbert *et al.* 1991b; Bompensieri *et al.* 1996). Although it was not within the scope of this study, it would be of interest to confirm the presence of a protease by zymogram analysis of supernatant proteins in future experiments.

The decrease in activity observed in trilaurin and tripalmitin cultures was in sharp contrast to that observed in hexadecane, where LipA is stable for more than 24

hrs at 30°C (Fig. 2-1) and for several days in crude supernatants stored at 4°C (not shown). The increase in stability in the presence of hexadecane may be attributed to association and protection of the lipase with hexadecane micelles and/or the lack of proteolysis in this medium. Overall, growth of RAG-1 in the presence of hexadecane elicited strong lipase production and promoted enhanced enzyme stability, providing a good medium for production and purification of the protein.

Purification of LipA

RAG-1 extracellular lipase was purified from stationary phase cells grown in LNPS plus 10mM hexadecane as the sole carbon source. Under these conditions, lipase accumulates in the medium with no apparent loss in activity. Specific lipase activity in filtered supernatants varied from 15 – 40 U/mg protein. LipA was purified with a 22% yield and greater than 10-fold purification. A summary of the purification scheme is presented in Table 2-2.

Preliminary binding experiments using ion exchange resins showed that LipA has no affinity for the cation resin tested (sulfopropyl, S) and only a small affinity (10%) for the anion matrix (trimethyl aminoethyl, Mono Q). Both matrixes were examined in various buffer systems (pH 5 – 9) and in the presence and absence of Triton X-100. Lack of affinity for ion exchange resins has been reported by others and attributed to enzyme aggregation (Breuil and Kushner 1975b) and association with LPS or other lipophilic components. (Stuer *et al.* 1986). While not effective in binding LipA, application of culture supernatant to Mono Q resins produced greater than two-fold purification of the lipase by effectively removing contaminating

Table 2-2. Purification of LipA from *Acinetobacter* sp. RAG-1.

Purification Method	Total Protein (mg)	Total activity (U)	Specific activity ($U \cdot mg^{-1}$)	Purification factor (fold)	Yield (%)
Concentrated Supernatant[2]	20.6	794	38.5	1	100
Mono Q	6.7	615	91.8	2.4	77.5
Butyl Sepharose	0.4	178	412	10.6	22.4

[1]Lipase activity (U) • mg^{-1} protein
[2]Two liters of filtered supernatant were concentrated ten-fold to 200 ml.

proteins. In addition, the method appears to be a suitable replacement for a lengthy decreasing NaCl gradient during HIC preceding elution with detergent (Bompensieri *et al.* 1996; Kok *et al.* 1995a).

LipA was effectively bound at room temperature to the Butyl Sepharose gel at approximately 0.25M NaCl in Buffer 1. The ability of LipA to bind hydrophobic matrixes under low salt concentration suggests the lipase is a strongly hydrophobic protein. Binding efficiencies of the matrixes varied (between 70% -90% of total activity bound), dependent upon gel usage. RAG-1 lipase remained tightly bound under conditions of decreasing NaCl concentration (not shown), but was effectively eluted by a second gradient of increasing concentraton (0 – 1%) of Triton X-100 (Fig. 2-3). A small "shoulder peak" in lipase activity prior to the main activity peak was sometimes seen during elution of LipA. Fractions collected from this portion of the gradient were found to contain other proteins in addition to LipA, presumably a result

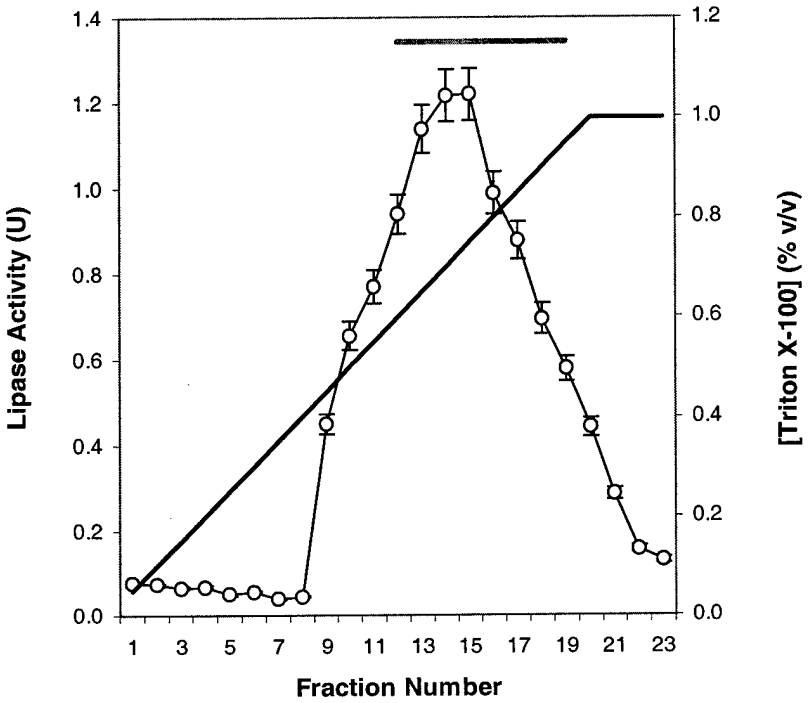


Fig. 2-3. Elution of LipA during hydrophobic interaction chromatography. LipA was eluted from butyl sepharose gel matrix under increasing concentration of nonionic detergent, Triton X-100 (—). Fractions (8 ml) were collected at 0°C and assayed for lipase activity (○) using *p*NPP substrate, as described in the text. Data are shown as the means of three determinations ± SE. Fractions collected and analyzed for purity using SDS PAGE are shown under the heavy gray bar.

of protein-protein interactions inherent in HIC (Scopes 1994). For this reason, only fractions near peak activity or later were collected and, on occasion, a second separation was performed on butyl sepharose.

Figure 2-3 shows LipA begins to elute from the column at 0.4% Triton X-100, followed by an activity peak at 0.7 % detergent. Fractions bordering the main activity peak were pooled and examined for purity by SDS-PAGE. Only a single major protein band, whose molecular mass (approximately 33 kDa) was consistent with the molecular weight deduced from the nucleotide sequence of *lipA*, was observed in

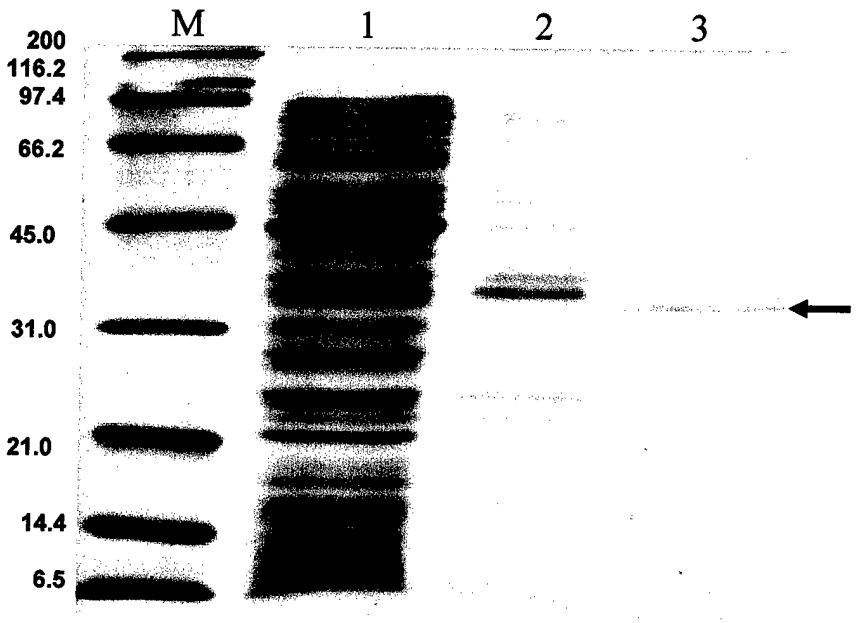


Fig. 2-4. SDS PAGE of extracellular lipase purified from *Acinetobacter* sp. RAG-1 grown in LNPS medium ammended with 10mM hexadecane. Separation was performed in 4% stacking gel and 15% separation gel with 5 M urea and 0.1% (w/v) SDS. Samples of mixed proteins (crude supernatant, ion exchange) contained 50μg protein per lane while highly purified samples contained 10μg lyophilized protein per lane. Protein bands were stained with Coomassie brilliant blue. Lane M, molecular weight markers; lane 1, concentrated supernatant; lane 2, sample after ion exchange; lane 3; purified LipA (arrow).

these fractions (Fig. 2-4). Experiments utilizing alternative detergents (CHAPS) in the elution buffer were successful in eluting the lipase from the hydrophobic matrix but with a compromise in purity. A smearing band in the gels in the 66kDa range was frequently observed, presumably due to the presence of residual emulsan present throughout the purification. It is possible that LipA may associate with the fatty acid component of the heterpolysaccharide.

Detergent removal and concentration determination

In many instances, it was desirable to remove residual Triton X-100 from protein samples; e.g., prior to gel electrophoresis, gel filtration, etc., and to determine detergent concentrations. While Triton X-100 is a powerful non-ionic detergent (CMC 0.02%), it forms high MW micelles and is non-dialyzable so must be removed by alternative means. To remove excess detergent, protein samples were incubated overnight at 4°C in the presence of Amberlite XAD-2 (Sigma) equilibrated in 30 mM Tris HCl, pH 8. This technique is effective in removing up to 50 mg detergent per gram (wet) absorbent with little loss (by absorption) of lipase activity. Recovery of active lipase was achieved by removing the absorbent by centrifugation.

To monitor detergent removal, a simple assay for determining Triton X-100 concentration in protein samples was developed. The method is based on the differential absorption between proteins and Triton X-100 at 289 nm. Wavelength scans of both protein (BSA) and Triton X-100 showed there was no single wavelength in which the detergent absorbed to the exclusion of protein (not shown). Instead, 289 nm was selected, based on the strong absorbance characteristics of the detergent and limited absorbance attributed to protein. A standard curve was prepared and Abs_{289} was found to be linear from 0.01 – 0.3% Triton X-100 (Fig. 2-5).

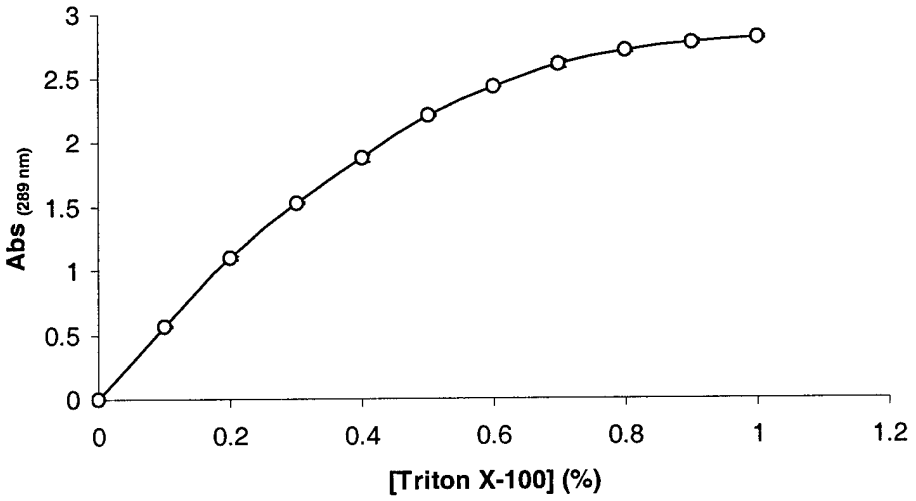


Fig. 2-5. Triton X-100 standard curve. Various concentrations (%, v/v) of Triton X-100 were prepared in 30 mM Tris HCl, pH 8 and Abs $_{289\ nm}$ determined. The curve was found to be linear from 0 – 0.3% detergent. Data are presented as the means of three replicates. Standard error was found to be ≤ 1%.

Gel filtration chromatography

Column fractions from HIC that contained the highest lipase activity were concentrated and applied to a Bio-Gel P60 (Bio Rad) gel filtration matrix (3,000 – 60,000 MW fractionation range) and eluted with 30 mM Tris-HCl/0.01% Triton X-100, pH 8. Under the conditions described, LipA eluted shortly after the void volume, indicating a MW between 35,000 – 60,000 (Fig. 2-6). This result was unexpected, as MW estimations by SDS-PAGE showed LipA to be 33 kDa. Similar results were obtained with Sephadex G-100 (Sigma), with larger fractionation range (not shown). Occurrence of this phenomenon is not uncommon, with alternative explanations provided. Elution directly after the void volume (Finkelstein *et al.* 1970) and variable elution due to aggregation (Gilbert *et al.* 1991a) were reported during gel filtration purifications of *P. aeruginosa* lipases. Alternately, hydrophobic

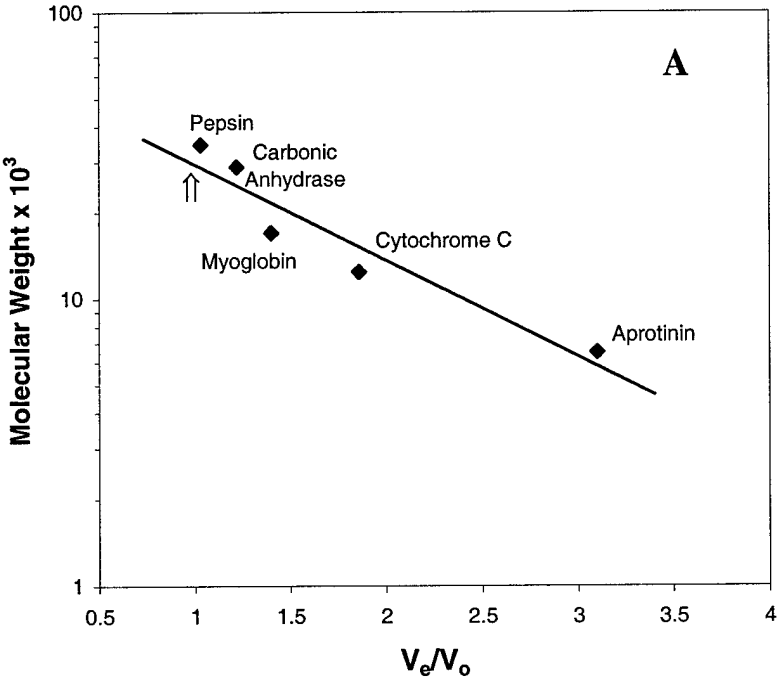
adsorption to the gel matrix may occur during elution, as reported for *Pseudomonas* sp. KWI-56 lipase (Iizumi *et al.* 1990).

Although aggregation and adsorption are plausible explanations for the overestimation in the size of LipA, it is also possible that the purified lipase associated with the detergent micelles during elution, with the same result. It is unfortunate that attempts to perform size exclusion chromatography in the absence of Triton X-100 resulted in inactivation of the protein. However, this may indicate that LipA requires a lipophilic component for activity. Recovery of enzyme following gel filtration was variable, between 50 – 75% yield.

Non-denaturing gel electrophoresis and zymography

Protein samples containing purified LipA were concentrated to ≥2.5 units lipase activity and separated on a 6% polyacrylamide gel under non-denaturing conditions. Gels were assayed for active lipase prior to staining by incubating between olive oil ($C_{18:1}$) and tricaprylin ($C_{8:0}$) emulsions (in agarose) for 4 – 16 hr at 37°C. Gels stained by Coomassie blue revealed the presence of a single band containing high

Fig. 2-6. Determination of LipA MW by size exclusion chromatography. (**A**). Column calibration curve determined using gel filtration standards (Sigma) eluted with 30 mM TRIS-HCl/0.01% Triton X-100, pH 8.0 on a 35 ml glass column (Econo-Pac, Bio Rad) packed with Bio-Gel P-60 gel (Bio-Rad) equilibrated in the same buffer. Protein samples were run in the cold (4°C) at 0.06 ml min^{-1}. Standard proteins: pepsin, hog, MW 34,700; carbonic anhydrase, bovine, MW 29,000; cytochrome C, horse heart, MW 12,400; aprotinin, bovine lung, MW 6,500. Void volume (V_o) was determined by elution of thyroglobulin (bovine), MW 669,000. (**B**). LipA fractions collected during HIC were pooled and concentrated to 200 µl, syringe-loaded, and eluted as in (A). Fractions (1 ml) were collected, elution volume (V_e) determined, and assayed for lipase activity (•) using *p*NPP as the substrate. The position of the void volume is indicated by (⇓).


A
100
10
1
Molecular Weight x 10^3
Pepsin
Carbonic
Anhydrase
Myoglobin
Cytochrome C
Aprotinin
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1
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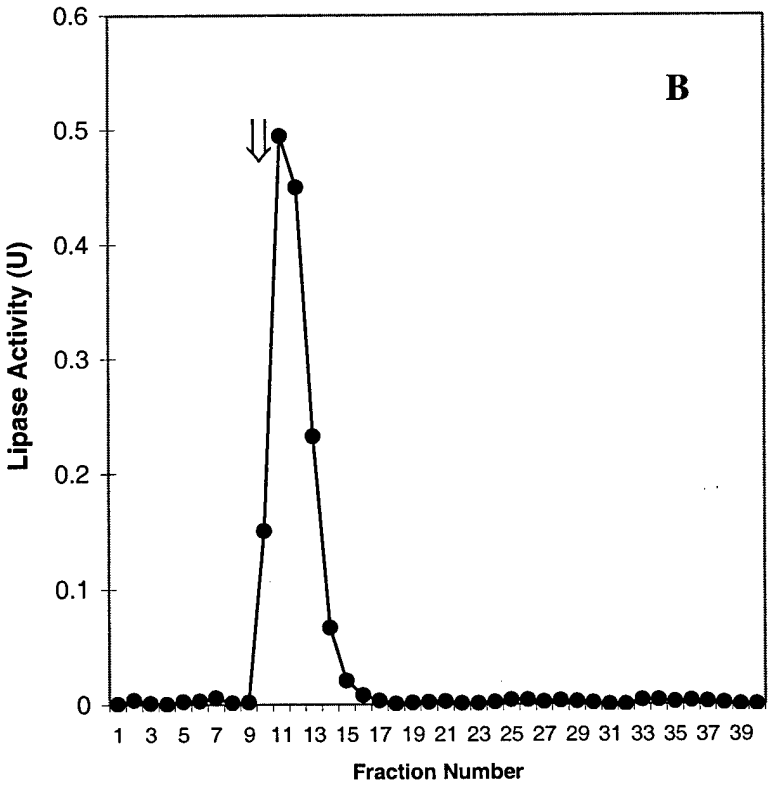
2.5
3
3.5
4
V_e/V_o


B
0.6
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Lipase Activity (U)
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39
Fraction Number

lipolytic activity (Fig. 2-7). The results show LipA is active towards emulsions of medium and long chain triglycerides. Areas of olive oil and tricaprylin hydrolysis are clearly shown and correspond with the protein band stained in native-PAGE gel (not shown). In accordance with guidelines proposed by Jaeger (Jaeger *et al.* 1999b) in defining lipases, the ability of LipA to hydrolyze long-chain triglycerides merits its classification as a true lipase (E.C.3.1.1.3).

During these experiments, we found LipA demonstrated a tendency toward aggregation. A small quantity of protein often did not enter the gel and a corresponding positive indication of lipase activity was detected in those areas of the zymograms (not shown in Fig. 2-7). Aggregation may be caused by residual detergent present in the samples or by precipitating lipase molecules in the stacking gel. These results are consistent with other lipase purifications where aggregation was reported to be a common phenomenon (Lesuisse *et al.* 1993; Hong and Chang 1988; Lin *et al.* 1996; Gilbert *et al.* 1991a).

Isoelectric focusing experiments showed the pI of LipA was 5.9 (not shown), in close agreement with the predicted value of 6.2 (Sullivan 1999).

Chapter summary

A. calcoaceticus RAG-1 cells were found to produce large quantities of extracellular lipase when grown on hexadecane as the sole carbon source. The lipase was secreted into the growth medium during the transition to stationary phase. It was found to be extremely stable in this medium, with no apparent loss of activity after 48 hr at 30°C. The enzyme is hydrophobic in character, as elution from hydrophobic

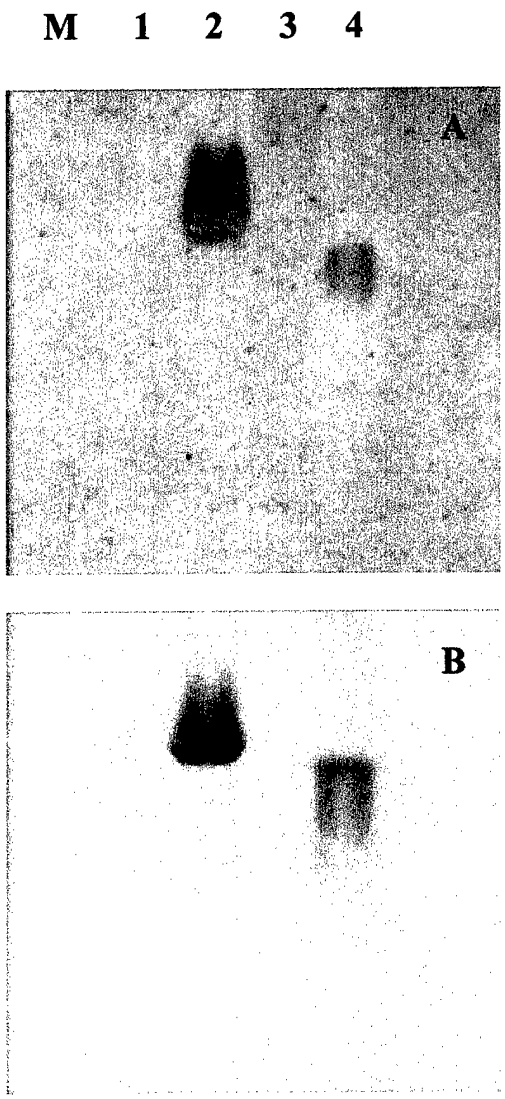

Fig 2-7. Zymograms demonstrating LipA activity toward olive oil (**A**) and tricaprylin (**B**) emulsions. Native PAGE gel was incubated between gels A and B for 16 hr at 37°C in a sealed, humid chamber. A positive indicator of lipolysis is demonstrated in (A) by the release of oleic acid and its interaction with the pH indicator (Victoria Blue B) and in (B) by tricaprylin hydrolysis (zone of clearing). Lane M, mixed marker proteins (neg. control); lane 1, crude supernatant after ion exchange (0.06 Units); lane 2, purified LipA (0.3 Units); lane 3, blank; lane 4, lipase from *Chromobacterium viscosum* (Sigma), (0.25 Units, pos. control).

gels was possible only by inclusion of Triton X-100 (or other detergents) in the elution buffer. RAG-1 lipase was purified, with a 22% yield and greater than 10-fold increase in specific activity. The lipase had an apparent molecular mass of 33 kDa, as determined by SDS PAGE. The enzyme showed a tendency toward aggregation, either as an artifact of the purification method (association with detergent micelles), or with some lipophilic component of the bacterium (emulsan or LPS). RAG-1 lipase had a pI of 5.9, as determined by isoelectric focusing. The lipase was active toward triglycerides and long acyl chain fatty acids and is, therefore, considered to be a true lipase.

Chapter 3. CHARACTERIZATION OF LIP A

CHAPTER ABSTRACT

An extracellular lipase, LipA, extracted from *Acinetobacter* sp. RAG-1 grown on hexadecane was purified and properties of the enzyme investigated. LipA was found to be stable at pH 5.8 – 9.0, with optimal activity at 9.0. The lipase remained active at temperatures up to 70°C, with a 3-fold increase in activity observed at its temperature optimum of 55°C, above that at 30°C. LipA is active against a wide range of fatty acid esters of *p*-nitrophenyl, but preferentially attacks medium length acyl chains (C_6, C_8). RAG-1 lipase is stabilized by Ca^{+2}; no loss in activity was observed in preparations containing the metal, compared to a 70% loss over 30 hr without Ca^{+2}. The lipase is strongly inhibited by EDTA, Hg^{+2}, and Cu^{+2}, but remains active after incubation with other metals or inhibitors examined in this study. The protein retains more than 75% of its initial activity after exposure to organic solvents, but is rapidly deactivated by pyridine. The characteristics described here suggest RAG-1 lipase offers great potential for use as a biocatalyst in organic synthesis. In addition, the properties of LipA suggest it should remain highly active in petroleum polluted environments, resembling that from which RAG-1 was originally isolated.

INTRODUCTION

Lipases (E.C. 3.1.1.3) are widely distributed in organisms, where they naturally catalyze the hydrolysis of glycerol esters to free fatty acids and mono- and diacylglycerols, many showing broad substrate specificity (Jaeger *et al.* 1994).

Lipases have been known to hydrolyze substrates in a highly stereo-selective manner in water, but they are increasing being recognized for their ability to catalyze transesterification, esterification, aminolysis, and acyl exchange reactions under nearly anhydrous conditions (Zaks and Klibanov 1985). Traditional substrates for these reactions include prochiral or chiral alcohols and carboxylic acid esters; but more recently, have been expanded to include diols, α-, β-hydroxy acids, lactones, amines, and cyanohydrins (for a complete review, see Jaeger and Reetz, 1998). Transformations involving a variety of substrates and high stereo-selectivity are two primary reasons why lipases have become very promising tools for the organic chemist. As the range of substrates and reactions catalyzed by lipases expands, the search for lipases with different biochemical properties that make these reactions more efficient and cost productive has also increased proportionally.

Despite considerable sequence similarities and the highly conserved α/β folding among bacterial lipases (Ollis *et al.* 1992), lipolytic enzymes display a wide variety of biochemical properties. As a result, only one is considered canonical in defining the enzyme class: hydrolysis of long-chain fatty acid esters (Jaeger *et al.* 1999b). Because of this diversity, reviews of these properties are not comprehensive, although several thoroughly discuss those of specific lipase families (Gilbert 1993; Svendsen *et al.* 1995) or representative enzymes across families (Jaeger *et al.* 1994; Arpigny and Jaeger 1999; Jaeger *et al.* 1999b). Following these examples, the biochemistry of lipase subfamilies I.1 and I.2 will be reviewed here as they pertain to the function and utility of the enzyme class.

Few bacterial lipases show optimum activity over a wide pH range. The most widely studied, those purified from *Pseudomonas* spp., predominantly demonstrate greater activity under slightly alkaline conditions (pH 8 – 9) (reviewed by Gilbert 1993). Few are active below pH 4, probably due to titration of the active-site histidine (Svendsen *et al.* 1995). Lipases that are highly active and stable in alkaline conditions are considered for use as detergent additives, where washing takes place near pH 10 (Jaeger and Reetz 1998).

Thermo-stability of enzymes is considered to be the most important characteristic justifying their use in industry (Herbert 1992). Many bacterial lipases are stable up to 50°C, with some of those purified from pseudomonads demonstrating stability at temperatures exceeding 60°C (Fox and Stepaniak 1983; Bozoðlu *et al.* 1984; Ilzumi *et al.* 1990). Resistance to heat denaturation is greatly enhanced by lyophilization, where the increased molecular rigidity attained is beneficial to protein stability (Klibanov 1989). Lyophilized porcine pancreatic lipase has been reported to be stable and active for several hours at 100°C in organic solvents, while in water inactivation occurs quickly at much lower temperatures (Zaks and Klibanov 1984; Zaks and Klibanov 1988).

All bacterial lipases used in defining subfamilies I.1 and I.2 contain aspartate residues that purportedly participate in Ca^{+2} binding (Arpigny and Jaeger 1999). The calcium ligand has been proposed to stabilize tertiary structure (Noble *et al.* 1993) and may be required for enzyme activity (Yang *et al.* 2000). However, supporting biochemical evidence for these roles is mixed. Calcium has been shown to increase activity in some I.1/I.2 lipases (Gilbert *et al.* 1991a; Kok *et al.* 1995a), but to have no

effect in others (IIzumi *et al.* 1990; Kim *et al.* 1996). Further, Ca^{+2} inhibition of lipase activity has also been demonstrated (Finkelstein *et al.* 1970). Despite these disparities, Ca^{+2}-binding seems to play a fundamental role in lipase structure, as its presence in all crystallized bacterial lipases suggest (Noble *et al.* 1993; Lang *et al.* 1996; Schrag *et al.* 1997; Kim *et al.* 1997). However, the biotechnological potential of some lipases may be limited by strict metallo-requirements in applications requiring the presence of chelating agents (detergent formulations) but would be of little consequence in others (organic synthesis).

In this study, the objectives of characterizing RAG-1 lipase were three-fold. First, anticipating an increased interest in lipases for use in industrial, pharmaceutical, and agricultural applications, the search for enzymes capable of performing the vast array of catalytic reactions proposed has accelerated. A thorough understanding of the biochemical properties of these enzymes is fundamental to assessing their potential and expanding their utility in these applications. RAG-1 lipase appears to have unique characteristics that contribute toward this goal. Second, phylogenetic comparison of LipA with lipases from *Pseudomonas* and *Burkholderia* families confirms its affiliation by sequence homologies (Sullivan *et al.* 1999). It is of interest to determine if the biochemical properties of RAG-1 lipase also closely resemble of related lipases already purified, providing further evidence for unity within the group. Third, a complete characterization of lipase properties is required to understand its activity and function as an extracellular enzyme secreted by cells in response to hydrocarbon exposure (Chapter 2). Design of experiments that examine the role of LipA in emulsan synthesis or modification, and emulsification production requires a

complete understanding of conditions promoting stability and activity. Properties such as substrate specificity, temperature/pH optima, and metallo-dependence may elucidate essential conditions required for enzymatic exchange of fatty acids residues on the polymer or other physiochemical relationships between the two biomolecules.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and lipase production

Acinetobacter sp. RAG-1 (ATCC 31012) was obtained from the American Type Culture Collection (Rockville, MD, USA). Cells recovered from frozen stock (-80°C) were grown on a minimal medium containing low nitrogen-phosphorus-sulfur (LNPS) content with 10 mM hexadecane as previously described by Leahy (Leahy 1993). RAG-1 has been shown to produce high volumetric extracellular lipase activity when grown on this medium (Leahy 1993; Sullivan 1999). Under these culture conditions, optimal specific lipase activity (U mg^{-1} extracellular protein) is detected in stationary phase (this work).

Lipase Purification

Culture supernatants containing extracellular lipase were harvested by pelleting stationary phase RAG-1 cells (10,000 x g) at 4°C for 10 min. Supernatants were pooled, filtered, and concentrated as described previously (this work). $CaCl_2$ and $MgCl_2$ (2 mM) were added to increase stability of the enzyme during purification. Precipitants and membrane-bound vesicles were removed by ultracentrifugation at

28,000 rpm (141,000 x g) for 1 hr at 4 °C (Sullivan 1999). Supernatants were divided into 35 ml aliquots and stored at -80°C prior to protein purification.

Samples were dialyzed overnight against 20 mM TRIS-HCl buffer (pH 8.0), followed by passage through two Econo-Pac Mono Q cartridges (Bio-Rad, Hercules, CA) fitted in tandem at approximately 1 ml min^{-1} to remove other supernatant proteins. Supernatant was mixed with an equal volume of HIC binding buffer (Buffer 1, 30 mM TRIS-HCl; 2 mM $CaCl_2$; 2 mM $MgCl_2$; 0.5 M NaCl) and mixed with butyl sepharose hydrophobic resin (Amersham Pharmacia Biotech, NJ). The mixture was allowed to stand at RT for approximately 30 min until maximum lipase saturation of the matrix occurred. The mixture was degassed, loaded onto a 25 ml glass column, and allowed to gravity pack. Hydrophobic interaction chromatography was performed as described elsewhere (Bompensieri *et al.* 1996; Kok *et al.* 1995a). Hydrophobic proteins were eluted by a gradient of 10 column volumes of Triton X-100 (0 – 1.0%) in 30 mM TRIS-HCl pH 8.0, followed by an additional 60 ml 1% Triton X-100. Fractions (8.0 ml) were monitored for protein content by UV absorption at 280 nm, collected on ice, and assayed for lipase activity using *p*NPP as the substrate. Fractions containing high lipase activity were pooled and tested for purity by SDS-PAGE. Aliquots (4 ml) for characterization experiments were concentrated using Ultrafree-4 centrifugal filter unit (10,000 MW cut-off, Millipore) and stored at –80°C. Lyophilized protein samples were prepared by freezing and then were dried over night at 7 microns Hg and -60°C.

Lipase Assay

Lipase activity was measured as hydrolysis of *p*-nitrophenyl palmitate (*p*NPP) in deoxycholate buffer, as described by Leahy (Leahy 1993). Samples (20 µl – 500 µl) were added to pre-warmed (30°C) phosphate buffer (50 mM, pH 8) containing 0.2% sodium deoxycholate and 0.1% gum arabic, final volume 3.0 ml. The mixture was incubated for 5 min at 30°C. *p*NPP (0.30 mM final concentration) was added and the mixture shaken, allowing the reaction to proceed for 3 min. Lipase activity was determined by the rate of *p*-nitrophenol production, measured at 405 nm in a model DU640 spectrophotometer (Beckman Coulter, Fullerton, CA). The reaction rate was calculated from the slope of the absorbance curve, using software installed by the manufacturer (Beckman Coulter). One unit of enzyme activity is defined as the amount of enzyme forming 1 µmol of *p*NP min^{-1} ml^{-1}. When examining the effect of temperature on activity, enzyme preparations were incubated at different temperatures for 5 min and assayed at the incubation temperature.

Measurement of protein concentration

Protein concentrations in samples containing less than 0.06% (v/v) Triton X-100 were measured by the method of Bradford (Bradford 1976) using bovine serum albumin (BSA) as the standard. Samples containing more detergent were assayed for protein concentration at 562 nm using the BCA protein assay kit (Pierce, Rockford, Il), using BSA as the standard. Samples containing up to 5% Triton X-100 could be assayed with BCA.

Effect of pH on lipase activity and stability

Activity toward *p*NPP at different pH values was determined using concentrated lipase preparations diluted four fold in each of several phosphate buffers (50 mM, pH 5.0 – 11.0). Mixtures were incubated in the appropriate buffer at 30°C for 1 hr. Lipase activity was determined in the same buffer adjusted to the incubation pH. The assay was performed using a sample volume of 20 µl and results were expressed as the relative percentage (mean of three determinations) of maximal activity. To determine the effect of pH on enzyme stability, concentrated lipase preparations were diluted four-fold in various buffers and incubated for 24 hr at 20°C. Buffers (50 mM) used were sodium acetate (pH 5.0 – 5.6), TRIS-malate (pH 5.8 – 7.5), TRIS-HCl (pH 7.5 – 8.5), 2-amino-2-methyl-1, 3-propanediol (pH 8.2– 9.5), glycine-NaOH (pH 8.6 – 10.6). Remaining activity (%) was measured under standard conditions using 20 µl sample volumes.

Substrate specificity

The specificity of LipA for different acyl chain lengths was examined using various esters of *p*-nitrophenyl (*p*NP). Assay conditions were identical to those described above, with the exception of substrate substitution. Nitrophenyl esters and associated acyl group chain lengths examined were as follows: pNP acetate (C_2); *p*NP butyrate (C_4); *p*NP caproate (C_6); *p*NP caprylate (C_8); *p*NP caprate (C_{10}); *p*NP laurate (C_{12}); *p*NP myristate (C_{14}); *p*NP palmitate (C_{16}); and *p*NP stearate (C_{18}). Specificity was examined under standard assay conditions using 20µl enzyme solutions in 30 mM TRIS-HCl buffer, pH 8.0. The reaction was started by adding 36 µl substrate

stock solution (in acetone, final concentration, 0.3 mM) and allowed to proceed for 3 min.

Effect of Ca^{+2} on lipase stability

Results of *lipA* sequence analysis in our laboratory suggested that the extracellular lipase from *Acinetobacter* sp. RAG-1 is capable of sequestering Ca^{+2} ions that may serve to stabilize the enzyme (Sullivan *et al.* 1999). However, the importance of Ca^{+2}-sequestering to enzyme stability in LipA was not investigated. Preliminary observations showed the enzyme was stable for extended periods of time (> 48 hr) at room temperature in the presence of Ca^{+2}. We chose to examine further the effect of Ca^{+2} on stability by incubating LipA in the presence and absence of Ca^{+2} and examining effects over time. Concentrated lipase in 30 mM TRIS-HCl, pH 8.0 (approximately 3.5 U) was dialyzed overnight against 1 liter 50 mM TRIS-HCl (pH 8.0). After dialysis, 20 µl aliquots of protein solution were diluted four-fold in 50 mM TRIS-HCl/2 mM $MgCl_2$, with and without addition of 2 mM $CaCl_2$ and incubated at 30°C. During a 30 hr period, experimental units (in triplicate) were selected and examined for lipase activity.

Sensitivity to inhibitors and organic solvents

LipA preparations were incubated with various compounds of potential inhibitory activity. Prior to incubation in the presence of these compounds, protein samples were dialyzed overnight against 30 mM TRIS-HCl pH 7.2. Lipase samples were diluted with stock solutions of inhibitors (final concentrations, 0.1 mM, 1.0 mM,

or 10.0 mM) and incubated at 30°C for 1 hr. At the end of the incubation period, residual activity was determined using *p*NPP as the substrate. Stability of LipA in organic solvents was measured similarly using lyophilized enzyme (0.01 mg) incubated in water miscible solvents (15% and 30%, v/v) in 30 mM TRIS-HCl (pH 8.0) for 1 hr at 30°C. Activity remaining (%) was measured under standard conditions.

RESULTS AND DISCUSSION

Purification of LipA

The purification protocol described in Chapter 2 was utilized for all chromatographic separations. Specific lipase activities measured by this procedure ranged from 400 –600 U/mg. Samples containing excess Triton X-100 were incubated in the presence of Amberlite XAD-2 (Sigma, St. Louis, MO) until detergent concentrations were reduced $\leq 0.05\%$ (v/v). The protocol for enzyme purification used here has the potential for lipase production on an industrial scale.

Effect of pH on lipase activity and stability

Figure 3-1A shows the effect of pH on the activity of LipA at 30°C with *p*-nitrophenyl palmitate as a substrate. The pH optimum was found to be approximately 9.0. The enzyme showed strong activity in the narrow pH range from 8.0 to 10.0 but activity decreased rapidly below pH 7.5 and above 10.5. No detectable lipase activity occurred at pH 5. These data are in good agreement with those from alkaline lipases reported for various strains of *Pseudomonas* (Choo *et al.* 1998;

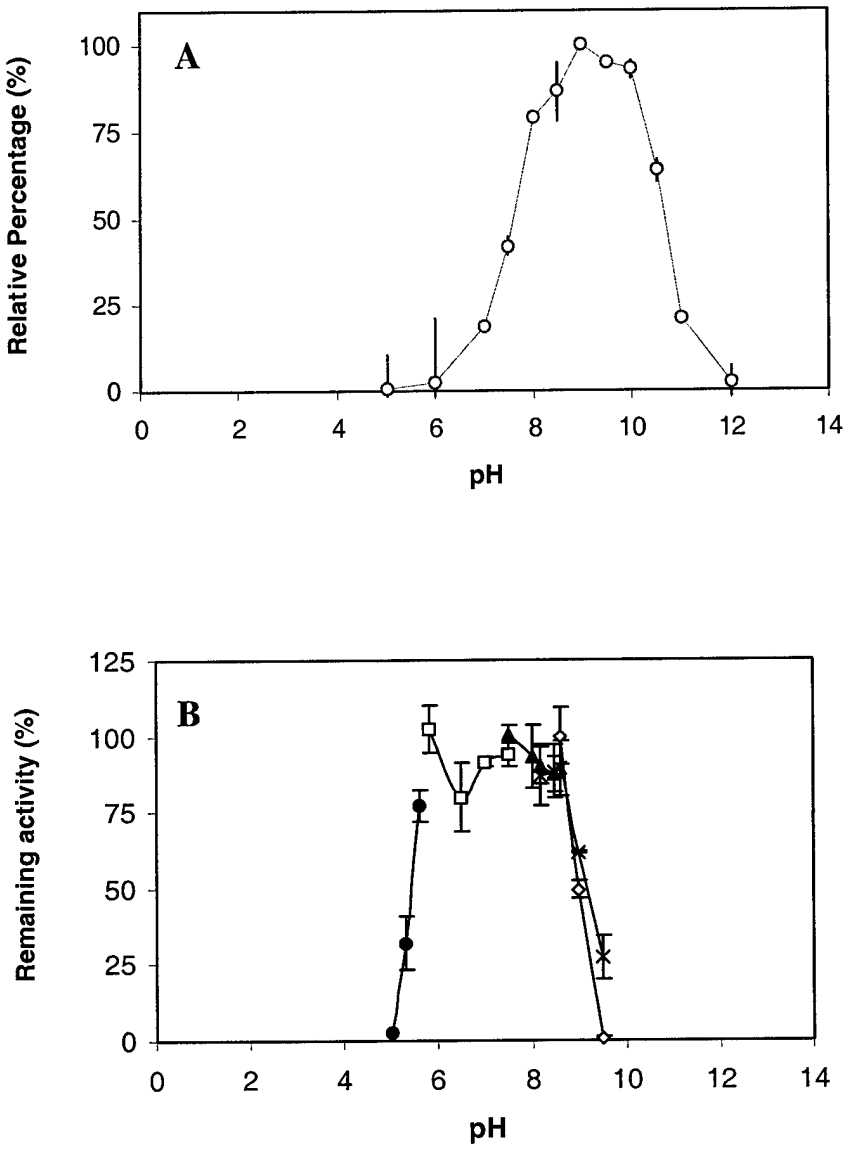

Fig. 3-1. The effect of pH on LipA activity (A) and stability (B). **A**. Concentrated protein samples were diluted four-fold in phosphate buffer (50 mM) set to the appropriate pH and incubated for 1 hour at 30°C. Lipase activity was assayed in the same buffer using *p*-NPP as the substrate. Results are given as relative percent of maximal activity (pH 9.0). **B**. Enzyme preparations were diluted four-fold in various buffers (50 mM) and incubated for 24 hours at 20°C. Remaining activity was assayed under standard conditions. Results are given as percentage of initial activity. (•), sodium acetate (pH 5.0 – 5.6); (□), TRIS-malate (pH 5.8 – 7.5); (▲), TRIS-HCl (pH 7.5 – 8.5); (×), 2-amino-2-methyl-1, 3-propanediol (pH 8.2– 9.5); (◊), glycine-NaOH (pH 8.6 – 10.6).

Gilbert *et al.* 1991a; Lin *et al.* 1996; Svendsen *et al.* 1995), *Acinetobacter* (Hong and Chang 1988; Kok *et al.* 1995a), and *Bacillus subtilis* 168 (Lesuisse *et al.* 1993).

In comparison, the pH stability curve of LipA showed the enzyme is stable under a wider pH range, but at slightly more acidic conditions (Fig. 3-1B). LipA retained 100% activity in the range pH 5.8 – pH 8.8, when incubated for a 24 hr period at 20°C. Below pH 5.6, stability of the molecule decreased sharply. The stability data suggest that the sharp decrease in activity below pH 6 (Fig 3-1A) was not because of poor stability (Fig. 3-1B), but possibly occurred during titration of the imidazole ring of the active site histidine. A similar mechanism of inactivation has been proposed for other serine hydrolases, e.g., serine proteases (Fersht 2000). Further, since significant activity was retained (80%) after incubation at pH 5.6, we suggest the dramatic reduction in stability under more acidic conditions (pH < 5.6) can be explained by loss of the Ca^{+2}-binding site, not by acid-induced denaturation. That is, incubation near pH 5 causes irrecoverable loss in stability by titration of coordinating Asp residues leading to Ca^{+2} release. This explanation is supported by results of structural studies employing X-ray crystallography (Noble *et al.* 1993).

Temperature

The enzyme had maximal activity at 55°C toward *p*-nitrophenyl palmitate (Fig. 3-2). At this temperature, LipA showed a three-fold increase in activity from that determined at the standard temperature (30°C). LipA remained very active at higher temperatures. Activities exceeding 1.5 times standard were achieved at

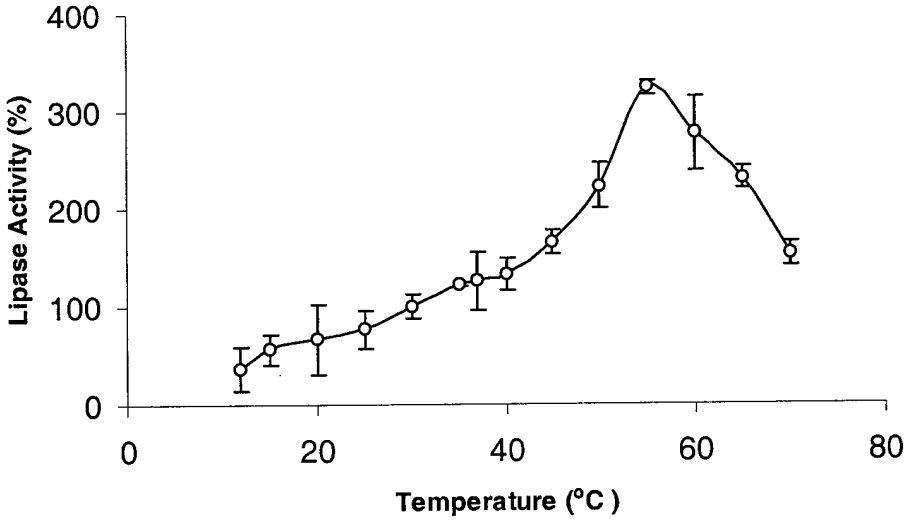

Fig. 3-2. Effect of temperature on the activity of LipA. The enzyme was incubated in 50 mM phosphate buffer (pH 8.0) for 5 min. at various temperatures and activity (○) ± SE determined in the incubation buffer using *p*-NPP as substrate. The value obtained at 30°C was taken as 100%.

temperatures up to 70°C. The temperature optimum reported here is higher than that reported for many bacterial lipases, although more thermo-tolerant examples exist in the genus *Pseudomonas* (Gilbert 1993). Activity at elevated temperatures is desired in biocatalysts used in biotechnology, because thermo-stable enzymes yield significant cost savings and the high temperatures can shift reaction equilibria in favor of the desired product (Herbert 1992). LipA remained stable for extended periods (> 48 hrs) at 25°C, with only small (10%) activity loss and could be stored for longer periods (5 d) at 4°C. In addition, RAG-1 lipase retained full activity after several cycles of freezing and thawing.

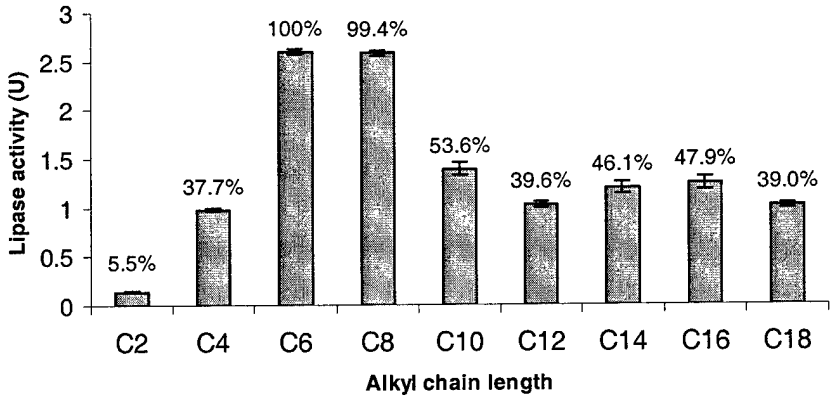

Fig. 3-3. Substrate specificity of LipA. Acyl-chain length specificity of purified LipA was determined from its activity toward various esters of *p*-NP (0.3mM). Percentages shown are relative to maximal activity (C_6). Data are means of three replications ± SE. Values were corrected against enzyme-free blanks.

Substrate specificity

Substrate specificity of LipA was examined using various fatty acid esters of *p*-nitrophenyl. The enzyme showed activity toward a broad range of acyl chain lengths but maximum activity occurred toward medium length (C_6 and C_8) fatty acid esters (Fig 3-3). Recent structural studies of the active site architecture in *P. cepacia* lipase revealed enantioselectivity may be determined predominantly by the size of the binding grooves (Lang and Dijkstra 1998). Therefore, the preference of LipA toward medium chain esters may indicate that the size of binding grooves of the catalytic site best accommodates medium chain-length esters. LipA has little esterolytic activity toward the more water-soluble substrate, *p*-nitrophenyl acetate (C_2). Similar preference for medium chain esters and triacyl glycerols has been reported in lipases purified from *Bacillus subtilis* 168 and *Aeromonas hydrophila* (Lesuisse *et al.* 1993;

Anguita *et al.* 1993). Broad-range substrate specificity is characteristic of many bacterial lipases (Jaeger *et al.* 1994) and is a primary reason for their enormous potential in biotechnology (Jaeger and Reetz 1998).

Lipases and esterases share common substrates in carboxylic acid esters. However, unlike esterases, lipases preferentially attack substrates that are water-insoluble, forming interfaces in aqueous solutions. Enzyme activation occurs by contact with the interface and is followed by catalysis between two phases (Brzozowski *et al.* 1991). Esters of medium and long-chain fatty acids are insoluble in water and, therefore, are preferentially hydrolyzed by true lipases (van Tilbeurgh *et al.* 1993; Derewenda and Sharp 1993). Although no evidence of interfacial activation is presented here, the data clearly show LipA is active toward a variety of water-insoluble esters and the specificity consistent with that of true lipases. Further, LipA is capable of hydrolyzing long acyl chain triglycerides (i.e., olive oil) used in zymogram preparation (Chapter 2). Additionally, comparative sequence analysis demonstrated overall similarity of *lipA* to other bacterial lipases (Sullivan 1999). Taken together, the biochemical properties and sequence data confirm classification of LipA as a true lipase.

Stability of LipA in the presence of Ca^{+2}

All bacterial lipases used in defining subfamilies I.1 and I.2 contain aspartate residues that purportedly participate in Ca^{+2} binding (Arpigny and Jaeger 1999). Despite the conservation of Ca^{+2}-binding residues in these lipases, response to Ca^{+2} addition has not been universal. Calcium has been reported to increase activity and

promote stability in lipases purified from *P. aeruginosa* EF2 (Gilbert *et al.* 1991a) and *Acinetobacter* sp. BD413 (Kok *et al.* 1995a), but have no effect on lipases purified from *Pseudomonas* sp. KWI-56 (IIzumi *et al.* 1990) or *Proteus vulgaris* K80 (Kim *et al.* 1996). Moreover, Ca^{+2} inhibition of lipase activity has been demonstrated in *P. aeruginosa* strain 10145 (Finkelstein *et al.* 1970). It should be noted that comparisons of the effect of Ca^{+2} relative to enzyme stability are difficult because of the variety of buffers as well as assay conditions used in the various studies. Despite these difficulties, Ca^{+2}-binding appears to play a fundamental role in lipase structure, as its presence in all crystallized family I.1/I.2 lipases suggests (Noble *et al.* 1993; Lang *et al.* 1996; Schrag *et al.* 1997; Kim *et al.* 1997).

We tested the hypothesis that Ca^{+2} may affect stability of LipA by incubating dialyzed lipase samples in the presence and absence of 2 mM $CaCl_2$ and examining effect over time (Fig. 3-4). The data clearly show that Ca^{+2} enhanced stability of RAG-1 lipase at 30°C. Over a 30-hr period, enzyme preparations without calcium lost up to 70% of initial activity. Those incubated in the presence of calcium retained 100% activity throughout the same period. Decreased stability in the absence of Ca^{+2} was also reported for a closely related lipase purified from *A. calcoaceticus* AAC323-1 (Bompensieri *et al.* 1996). Based on comparative biochemical evidence available, we suggest Ca^{+2} sequestering is conserved within subfamilies I.1 and I.2. However, it is highly probable that the strength of Ca^{+2} coordination varies, explaining the dissimilarities in results reported. Our results suggest that, in LipA, simple diffusion of Ca^{+2} out of the binding pocket occurs within as little as 4 hr (Fig. 3-4), indicating weak coordination of the metal. Loss in activity may be due to the concomitant

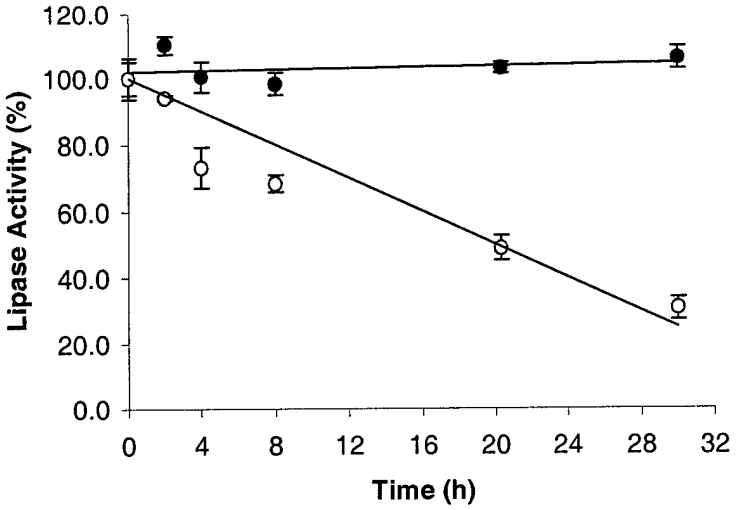

Fig. 3-4. Stabilizing effect of Ca^{+2} on LipA. Enzyme preparations were dialyzed against 30mM TRIS-HCl (pH 8.0) prior to incubation in 30 mM TRIS-HCl/2 mM $MgCl_2$ with (●) and without (○) 2mM $CaCl_2$. Replicates (3) were examined at various times over a 30 hr period and activity plotted as the percentage of initial activity.

disruption of the hydrogen-bonding network leading to the nearby active-site histidine (Fig. 1-4) (Noble *et al.* 1993), (Kim *et al.* 1997). The biotechnological potential of lipases with strict metallo-requirements may be limited in applications requiring the presence of chelating agents (detergent formulations) but would be of little consequence in others (organic synthesis).

Effects of cations and inhibitors

LipA was incubated at 30°C for 1 hr with various cations and inhibitors that have been shown to affect Group I (*Pseudomonas/Burkholderia*) lipases (reviewed by Gilbert 1993). The activity of LipA was increased approximately 20% by exposure to low concentration (1 mM) of $CaCl_2$ (Table 3-1). Incubation with 10 mM Ca^{+2}

Table 3-1. Effect of various inhibitors on LipA[1]

Compound	Remaining activity (%)[2] at a concentration (mM) of:		
	0.1	1.0	10.0
$CaCl_2$		122.9±1.1	104.6±1.1
$MgCl_2$		116.5±1.3	95.8±5.8
$MnCl_2$		98.0±8.3	117.3±6.1
$ZnCl_2$		110.0±1.5	86.3±5.8
$CuCl_2$		36.9±6.0	16.2±5.8
$CoCl_2$		140.7±6.2	117.8±7.1
$FeCl_3$	133.0±6.5	127.3±6.2	ND[3]
$HgCl_2$	85.2±1.1	11.9±0.9	ND
RbCl		150.1±6.3	130.4±8.5
EDTA		10.7±0.3	6.5±0.5
EDTA+Ca^{+2}		9.3±1.0	8.1±1.2[4]
PMSF	93.6±4.2	88.6±2.7	88.2±6.0
DTT		92.0±3.1	93.1±2.3
2-mercaptoethanol			94.7±2.2

[1]LipA was incubated with various compounds that may inhibit the enzyme, and the remaining activity was measured under standard conditions. Enzyme preparations were dialyzed against 30 mM TRIS-HCl pH 8.0 prior to the experiment. The samples were diluted with stock solutions of inhibitors to make the final concentration indicated and incubated at 30°C for 1 hr. The activity remaining was expressed as a percent of the appropriate control value (with no addition) post incubation.
[2]The activity remaining was expressed as a percent of the appropriate control value (with no addition) post incubation. Values shown are means of at least three replications ± SE.
[3]ND = not determined
[4]20mM $CaCl_2$ added post incubation with EDTA and incubated at 4°C for 20 hrs

resulted in only a small increase in activity. Exposure to low concentrations of other cations (Mg^{+2}, Co^{+2}, Fe^{+3}, and Rb^{+}) resulted in a similar increase in activity, while increasing metal concentration ten-fold had no effect. Zn^{+2} (10 mM), Hg^{+2} (1 mM) and Cu^{+2} inhibited the lipase, reducing activity by 15%, 88%, and 85%, respectively. Similar inhibition by heavy metals has been noted by other investigators (Anguita *et al.* 1993; Choo *et al.* 1998; Ilzumi *et al.* 1990).

EDTA strongly inhibited enzyme activity, with exposure at 1 mM resulting in 90% loss. This effect was found to be irreversible; no activity was recovered in these experiments post incubation with 20 mM $CaCl_2$ overnight at 4°C. Perhaps additional experiments may show the effect of EDTA can be mitigated if a lower pH is employed (Svendsen *et al.* 1995). Because the existence of a Ca^{+2}-binding site is compelling (Sullivan *et al.* 1999), these data indicate that stability and activity in LipA at even low temperatures is Ca^{+2}-dependent. Removal of Ca^{+2} by EDTA treatment may alter the three-dimensional structure sufficiently to prevent refolding upon reintroduction of Ca^{+2}. Further, because Ca^{+2} addition after EDTA treatment does not reactivate the enzyme, Ca^{+2} may be required during the folding process, perhaps for interaction with the foldase (LipB). Proper folding and activation has been shown to be influenced by Ca^{+2}-binding in *Pseudomonas* sp strain KWI-56 lipase (Yang *et al.* 2000).

It is difficult to interpret the effect (or combination of effects) of other divalent metals on lipase stability. At low concentrations, many enhanced stability, possibly by serving as suitable replacement for Ca^{+2} (Table 3-1). This has been found to be the case for Mn^{+2} in *Pseudomonas* lipase (Shibata *et al.* 1998). In addition, metals may also show variable affects on lipase aggregation or may modify the substrate-water interface through interaction with free fatty acids (Lesuisse *et al.* 1993) (Brockerhoff and Jensen 1974). Some combination of these effects is probably responsible for the increased stability observed in the presence of most of the cations examined in this study.

The role of the disulfide bridge in the production of active lipase is not well established. In *Pseudomonas* sp. KWI-56 lipase, a functional disulfide linkage is required to ensure correct folding during synthesis and secretion (Yang *et al.* 2000). Alternately, Liebeton *et al.* (2001) found the disulfide linkage in *P. aeruginosa* not to be required for interaction with its foldase, but to provide stability for the active conformation of the lipase (Liebeton *et al.* 2001). Further, the disulfide bridge may enhance stability by decreasing sensitivity of *Aeromonas hydrophila* lipase to intracellular proteases encountered during export (Brumlik *et al.* 1997).

The results presented here show LipA activity was unaffected by DTT or 2-mercaptoethanol. This indicates the putative disulfide bridge is not required for stability at low temperatures (30°C) and suggests an alternative role, perhaps in protein folding or protection from proteases. However, it is possible that the disulfide bond may not be accessible to the reducing agents, either as a result of its tertiary structure or by association with other compounds; i.e., LPS. Stuer (Stuer *et al.* 1986) demonstrated the association of *P. aeruginosa* lipase with LPS, and suggested it may prevent heat denaturation and confer resistance toward DTT. However, Brumlik *et al.* (Brumlik *et al.* 1997) proposed that stability of *Aeromonas hydrophila* lipase in the presence of DTT results from a deeply recessed location of the disulfide linkage. The fact that LipA shows a tendency to smear during native gel electrophoresis (Chapter 2) supports the former explanation (LPS association). Further, published three-dimensional structures of lipases of subfamilies I.1./I.2 show the surface location of the disulfide linkage (Noble *et al.* 1993), (Nardini *et al.* 2000; Schrag *et al.* 1997). Loss of the disulfide bond (by thiol reductants) in RAG-1 lipase was not

examined at higher temperatures or in conjunction with stronger denaturing agents (i.e., SDS, urea). It may be that under these conditions, the disulfide bridge becomes more accessible to reducing agents, revealing its importance in protein stabilization.

Lipases are members of the serine-hydrolase family of enzymes having a trypsin-like catalytic triad of Asp (Glu)-His-Ser, with the active-site serine serving as the nucleophile (Brady *et al.* 1990; Ollis *et al.* 1992). However, LipA exposure to the serine-reactive agent PMSF did not result in significant inhibition. This result is not unusual, since other bacterial lipases demonstrate similar resistance to such inhibitors in aqueous solutions (Kordel *et al.* 1991; Nakatani *et al.* 1992; Kim *et al.* 1996; Choo *et al.* 1998). Resistance to inactivation by PMSF may be explained either by the inaccessibility of the agent to the buried active site in the closed conformation (Noble *et al.* 1994) or by enzyme aggregation and association with LPS. Future experiments can be designed to show that accessibility to the active site (followed by inactivation) may be possible under conditions where the enzyme is activated (open conformation) in the presence of a substrate-water interface.

Stability of lipase (LipA) in organic solvents

Lyophilized LipA was incubated with a variety of water-miscible organic solvents for 1 hr at 30°C. Most of the solvents examined had little effect on lipase stability; 90% or greater activity was retained (Table 3-2). The enzyme lost less than 30% of its initial activity after incubation with acetonitrile. This solvent has been shown to completely deactivate lipases in concentrations as low as 15% (v/v) (Shabtai and Daya-Mishne 1992; Choo *et al.* 1998; Shimada *et al.* 1993). Pyridine caused

Table 3-2. Stability of LipA in selected organic solvents[1]

Solvent	Remaining activity (%)at[2] a concentration (v/v) of:	
	15%	30%
isopropyl alcohol	98.0±2.6	91.8±2.8
DMF	83.3±2.3	95.7±2.8
acetone	95.8±2.9	93.2±3.8
DMSO	99.6±0.8	90.8±2.4
THF	90.8±0.5	10.8±2.3
acetonitrile	72.5±0.6	77.6±8.4
pyridine	24.7±1.1	0

[1]Lyophilized lipase was incubated with various organic solvents for 1 hour at 30°C. Residual activity was measured using standard conditions described in text

[2]Results are expressed as the percentage of activity with no addition of solvent. Values shown are means of at least three replications ± SE.

significant deactivation; concentrations exceeding 15% (v/v) resulted in complete activity loss within 1 hr (not shown). Similar sensitivities to pyridine (Lesuisse *et al.* 1993; Shabtai and Daya-Mishne 1992) have been reported. The data suggest interaction with pyridine probably causes rapid denaturation of the lipase. Lyophilized LipA remained highly active in the presence of hexane inconcentrations up to 99.8% (v/v) (not shown). Overall, the enzyme showed marked insensitivity to solvents, a characteristic necessary for use of LipA as a biocatalyst in organic synthesis.

Chapter Summary

Acinetobacter sp. RAG-1 lipase was purified and characterized, with many of its properties being shared with those required for industrial biocatalysts. Stability of LipA was greatly enhanced by the presence of Ca^{+2}, a property that must be

considered in future applications. This characteristic appears to be conserved in lipase subfamilies I.1 and I.2. LipA showed low substrate specificity. It was active toward a variety of medium and long chain esters, another important characteristic of industrial lipases. It had high activity at elevated temperatures and was resistant to inactivation by many metals, inhibitors, and organic solvents. Resistance to these inhibitors may be derived, in part, from association with LPS.

Based on the properties described here, LipA appears to be an excellent candidate for used as a biocatalyst in selective transesterification reactions. This potential is explored further in Chapter 4. Moreover, because RAG-1 lipase maintained activity in the presence of the many inhibitors, cations, and solvents examined in this study, it is concluded that it would be effective in petroleum contaminated environments resembling that from which RAG-1 was originally isolated.

Chapter 4. ROLE OF RAG-1 LIPASE IN EMULSION PRODUCTION

CHAPTER ABSTRACT

The catalytic activity of lipase purified from *Acinetobacter* sp. RAG-1 was determined in organic media. Transesterification of 1-octanol with vinyl butyrate in hexane was used as the model reaction. Production of 1-octylbutyrate was monitored by GC analysis. The reaction was performed at various a_w, and initial reaction rates determined. Optimal a_w for ester synthesis was a_w 0.86, forming 18.9 μmoles hr^{-1} 1-octyl butyrate. LipA-catalyzed acyl transfer of emulsan fatty acids in organic media was also examined. Emulsan, the polyanionic emulsifier, contains covalently bound fatty acids that impart emulsifying activity to the polymer. Emulsan fatty acids were methylated (FAMES) and their composition analyzed by gas chromatography. Emulsan incubated in the presence of LipA showed significant reduction (mole %) in 2-hydroxy lauric and palmitic acids compared to controls experiments. However, no significant difference was found with respect to the transesterified fatty acid (octanoic acid). Emulsifying activity of emulsan mixed with various proteins was determined. Experiments containing LipA and emulsan increased emulsifying activity 2-fold over those observed in other emulsan/protein mixtures, emulsan, or LipA alone, suggesting a LipA-emulsan specific interaction. It is proposed that LipA may associate with emulsan through hydrophobic interaction with its lipophilic moiety. Fatty acid inhibition of LipA was also examined by adding dodecanoic (lauric) acid to assay mixtures. Lauric acid was not found to be an effective inhibitor of lipase but some interfacial inhibition was observed. These data suggest that LipA should remain

highly active in the presence of emulsan-bound fatty acids and support the hypothesis of a LipA-emulsan association.

INTRODUCTION

Many different microorganisms are capable of growth in media containing hydrocarbons. Among the bacteria, *Acinetobacter* and *Pseudomonas* spp. often predominate in oil-polluted waters and sediments (Blaise and Armstrong 1973; Walker *et al.* 1975). Further, many bacterial strains isolated from environments contaminated by hydrocarbons produce extracellular lipases (Breuil and Kushner 1975a; Breuil *et al.* 1978). Schindler (Schindler *et al.* 1975) first noted a correlation between the ability to utilize hydrocarbons and lipase production. An effect of crude oil on freshwater bacterial populations was selection for strains metabolizing hydrocarbons and these strtains also produced lipase. However, strains that showed no lipolytic activity did not utilize hexadecane. Breuil *et al.* (Breuil *et al.* 1978) found a similar result for *A. lwoffi,* strain O_{16} and *P. aeruginosa*, but also noted that production of lipase by other bacteria included in their study did not always guarantee utilization of hydrocarbons. *Acinetobacter lwoffi* strain O_{16}, originally isolated from polluted river sediment, showed lipase induction when grown on alkanes but the investigators were unable to identify the role of lipase in hydrocarbon degradation (Breuil and Kushner 1975a; Breuil *et al.* 1975; Breuil *et al.* 1978).

Although production of lipases by bacterial strains capable of hydrocarbon utilization is well established, the reason the enzyme is secreted in response to hydrocarbon exposure remains unsolved. Strong correlations between lipase

production and hydrocarbon utilization certainly seem to suggest that lipase is needed in some way for utilization of the alkanes (Schindler *et al.* 1975; Breuil *et al.* 1978). It is clear that lipases cannot act directly in hydrocarbon catabolism, as alkanes are not lipase substrates. It has been suggested that they play an indirect role in hydrocarbon degradation through the production of free fatty acids that serve to lower interfacial tension and thereby increase hydrocarbon uptake (Propkop *et al.* 1972). In support of this hypothesis, Breuil and Kushner (Breuil and Kushner 1980) demonstrated the addition of long chain fatty acids to a mixture of ethanol and hexadecane (as sole carbon sources) reduced lag phase and suppressed diauxic growth in *Acinetobacter lwoffi*. They attributed much of this effect to the emulsifying ability of the fatty acids, although palmitic acid was also used as a carbon source. Further, they postulated that palmitic acid produced by hexadecane metabolism or triglyceride hydrolysis may be directly incorporated into the membrane thereby aiding hydrocarbon uptake (Breuil and Kushner 1980).

Previous Research

Our laboratory has studied extracellular lipase production by *Acinetobacter* sp. RAG-1 cells grown on hexadecane and hypothesized that RAG-1 lipase acts to esterify free fatty acids to the emulsifier polysaccharide backbone, and may rearrange covalently-bonded fatty acids by trans-esterifications (Leahy 1993; Sullivan 1999). During the course of the research, we reported the sequence of LipA (Sullivan *et al.* 1999) and have established a correlation between its production and synthesis of emulsan (Leahy 1993; Sullivan 1999). Specifically, a linear relationship between

lipase activity and fatty acid content per mole emulsan and emulsification production was established (Leahy 1993). In addition, increased lipase activity was found to be commensurate with increased specific fatty acid content of the polymer (Leahy 1993). These data provided the basis for the hypothesis that RAG-1 lipase acts to directly bind fatty acids to emulsan (initial esterification) or to carry out post-synthesis modifications via trans-esterification reactions (Leahy 1993). Evidence supporting this view was several fold: 1) LipA$^-$ cells were found to have lower specific fatty acid content and emulsifying activity than wild type (WT) cells, 2) emulsans from a complement mutant strain (LipA$^+$, a lipase "overproducer"), contained greater fatty acid content and emulsifying activity than WT cells, and 3) lipase activity showed both positive and negative correlations with emulsan fatty acids (hexadecenoic acid and an unidentified fatty acid, respectively).

In testing this hypothesis, two separate studies of comparative fatty acid analyses of emulsan fatty acids were performed, yielding mixed results. Leahy (Leahy 1993) demonstrated both qualitative and quantitative differences in emulsan fatty acids from LipA$^-$ and WT cells grown in hexadecane, while Sullivan (Sullivan 1999) could not detect a significant difference between the two. The lack of agreement between these studies may be explained by either a lack of quantitative GC standards, improved harvest procedures used in the latest study, or different growth phases at time of harvesting cells (stationary vs. late logarithmic phase, respectively).

In reviewing previous strategies for testing the transesterification hypothesis, it is concluded that the thermodynamics of lipolysis was not fully considered in the experimental design. That is, experimental conditions were not optimum for

successful detection of transesterification products. As an hydrolase, lipases by nature catalyze reversible reactions (Fig. 1-1). However, while theoretically possible, lipase-catalyzed transesterification reactions in aqueous systems are not thermodynamically favored, and therefore, products of these reactions would probably remain undetected. In addition, other studies have shown emulsan synthesis is a dynamic and adaptive process, dependent upon growth and substrate conditions employed (Gorkovenko *et al.* 1997; Zhang *et al.* 1997). The fact that LipA$^-$ mutant strains produce emulsans that contain covalently bond fatty acids support these studies by suggesting the involvement of an additional acyl transferase in emulsan production and modification. Together, these data suggest alternative mechanisms may exist for synthesis and turn-over of the polymer fatty acid component. Further, they suggest *in vivo* experimental strategies for detecting fatty acid modifications may not be appropriate.

Experimental Strategy

In this study, we selected an *in vitro* approach in examining LipA catalyzed fatty acid modification of emulsan. We used only a single exogenously added fatty acid (caprylic acid, C_8) in the presence of LipA to detect transesterification rearrangements. In addition, we gave additional consideration to the thermodynamics and chemistry of lipolysis. We elected to perform experiments under nearly anhydrous conditions, allowing for minimal contribution by the back reaction (hydrolysis). It could be argued that these conditions (nearly anhydrous) do not normally exist in environments where acinetobacters are commonly found. However,

RAG-1 was originally isolated from oil contaminated seawater and is capable of degrading crude oil (Reisfeld *et al.* 1972). Sea borne oil commonly forms both water-in-oil and oil-in-water emulsions, the latter of which may contain up to 80% oil (Environment Directorate 1977; Office of Technology Assessment 1991). It is not improbable that emulsions exceeding this concentration may persist on a micro-scale or that emulsions formed near the source of contamination may contain a higher percentage of hydrocarbons for shorter time periods. We, therefore, consider the experimental design may be more applicable than previous studies to those conditions from which the bacterium was isolated.

Emulsan Analogs

Throughout this work two themes have emerged. First, understanding the conditions promoting lipase production and its role during growth of RAG-1 on hexadecane. A second area of focus was to assess the biotechnological potential of the newly purified lipase, LipA. Characteristics of lipases providing a vast biotechnological potential include (1) stability in organic solvents, (2) broad substrate specificity, (3) stereoselectivity during catalysis, and (4) lack of cofactor requirement (Jaeger and Reetz 1998). Of these, we have provided evidence for (1) and (2). These characteristics of LipA suggest not only that it may be a good candidate for further study in biotechnological applications, but may be desirable for its putative role in modifying emulsan fatty acids in the presence of organic solvents. Further, if this role of LipA is established, the lipase would be attractive for use in bioengineering emulsan analogs, each with unique properties and potential applications.

The pursuit of strategies that allow tailoring of emulsan structure is not without precedent. Of these, selective feeding of specific fatty acids to RAG-1 cells predominates. This method has yielded emulsans with enhanced emulsification properties toward specific substrates (Gorkovenko *et al.* 1997). Direct incorporation of exogenous hydroxylated fatty acids has been reported, but without detectable increase in emulsifying activity (Zhang *et al.* 1997). Cell-directed incorporation of exogenous fatty acids has also been shown under conditions limiting nascent fatty acid biosynthesis, yielding modified emulsan products with reduced emulsification properties (Kim *et al.* 2000). These studies clearly show that modification of the fatty acid structure does occur, but these changes are not always associated with an increase in emulsifying activity. Presumably, the emulsan analogs created by these methods possess other surface-active properties that remain undetected by the assay employed.

The strategy employed here is to create emulsan analogs in a cell-free system by enzyme-catalyzed acyl transfer of fatty acids to the emulsan polymer. In aqueous solutions, LipA shows broad substrate specificity ($C_4 - C_{18}$), but is most active against medium acyl chain lengths (C_6, C_8) (Fig. 3-3). In non-aqueous media, it is reasonable to expect LipA to show similar specificity, catalyzing the acyl transfer of natural emulsan fatty acids (chain lengths $C_{10} - C_{18}$) with exogenous free fatty acid (octanoic acid) present in molar excess. The method may allow bioengineering of a variety of emulsan analogs through lipase and free fatty acid selection.

MATERIALS AND METHODS

Transesterification reactions: general procedures

It is well established that enzyme catalytic activity in organic media is dependent upon the ionization state attained in water prior to precipitation or lyophilization (Zaks and Klibanov 1985; Zaks and Klibanov 1988). This is because ionogenic groups acquire different ionization states in aqueous media that are retained or "remembered" in the solid state (Zaks and Klibanov 1988). Therefore, dried or solid enzymes show a pH history, with optimum activity in organic media coinciding with that shown in water. Following this rationale, LipA samples were prepared for freeze drying by dialysis overnight against 30 mM Tris-HCl/5 mM $CaCl_2$, pH 9.0. Previous experiments demonstrated optimal enzyme stability and activity was attained in the presence of $CaCl_2$ and at pH 9.0 (Figs. 3-1, 3-4). Dialyzed samples containing the desired total protein were added to 4.5 ml glass sample vials and lyophilized overnight (7 microns Hg, -60°C). All reactants and solvents were dried in the presence of molecular sieves (4 Å) for a minimum of 48 hr before use. The lyophilized enzyme powder and organic phase were incubated (separately) for 48 hr in a sealed chamber containing saturated salt solution. Vapors from the salt solution equilibrated samples to a known water activity (a_w) as described by Valivety (Valivety *et al.* 1992). Salt solutions included $LiCl \cdot H_20$, (a_w 0.11); $MgCl_2\ 6H_20$, (a_w 0.33); $Mg(NO_3)_2 \cdot 6H_2O$, (a_w 0.55); NaCl, (a_w 0.75); (KCl, (a_w 0.84); and $K_2Cr_2O_7$, (a_w 0.97). Reactants and solvent were combined in glass sample vials containing lyophilized lipase and incubated at 40°C at 300 rpm for 30 hr.

Synthesis of octyl butyrate

Lipase-catalyzed transesterfication of vinyl butyrate with 1-octanol in *n*-hexane served as a model reaction for transesterification by alcoholysis:

vinyl butyrate + 1-octanol ⇄ 1-octyl butyrate + acetaldehyde
ester *alcohol* *product ester* *aldehyde*

Lyophilized lipase powder (10μg) was equilibrated over saturated salt vapors, as described above. All reactions were performed in triplicate in sample vials containing 1 ml hexane and 0.8M vinyl butyrate and 0.2M 1-octanol. Reaction vials were incubated for 30 hr at 40°C, 300 rpm (New Brunswick, Edison, NJ). Over the course of the incubation period, reaction mixtures were assayed for the production of 1-octyl butyrate by removing 1μl aliquots by glass syringe and diluting 1500-fold in hexane containing 0.266mM octadecane (internal standard). Samples were analyzed by gas chromatography (GC) using a Hewlett Packard (Palo Alto, CA) gas chromatograph, model HP 5890 and retention times compared with standard compounds (Sigma-Aldrich, St. Louis, MO). Optimum system water content and initial reaction velocities were determined from time course of 1-octyl butyrate formation by fitting product concentrations to a linear function via regression analysis.

Gas Chromatography

A Hewlett Packard gas chromatograph (model, HP 5890) fitted with an OMEGAWAX™ 320 (Supelco, Bellafonte, PA) glass capillary column (30m x

0.32mm id x 0.25 μm film thickness) attached to an FID (260°C) was used for all analyses. Helium was used as the carrier gas at a flow rate of 1.2 ml min^{-1}. Samples (1 μl) were applied to the column using splitless injections at 250°C. The separation method employed was as follows: initial oven temperature of 50°C for 1 min, 20°C min^{-1} ramp to 200°C, 20°C min^{-1} ramp to 240°C, final temperature for 5 min. Under the conditions described, the retention times for vinyl butyrate, 1-octanol, 1-octyl butyrate, and internal standard were 2.06 min, 5.93 min, 6.36 min, and 7.49, respectively. Acetaldehyde is assumed to volatilize in the reaction and was not detected by GC. Standard compounds (in hexane) purchased from Sigma (St. Louis, MO) were used to calibrate the column over a 15-fold range in concentration (33 μM, 66 μM, 133 μM, 266 μM, and 533 μM) and at a constant internal standard concentration (0.266 μM). Standard curves produced showed linear signal response with increasing concentration over this range.

Transesterification of emulsan

Previous studies describing the lipid moiety of emulsans harvested under a variety of growth conditions revealed that they are comprised of $C_{10} - C_{18}$ fatty acids covalently attached to the polysaccharide backbone by *O*-ester and *N*-acyl linkages (Belsky *et al.* 1979). Substrate specificity experiments described here demonstrated LipA activity toward a wide variety of *p*NP esters with maximum activity toward $C_6 - C_8$ acyl chain lengths (Fig. 3-3). We reasoned that similar substrate specificity may exist in organic media, and that a reasonable chance for successful transesterification and product detection exists using octanoic acid as the acylating agent. Further,

attachment of octanoic acid to the emulsifier should occur at the expense of one or more naturally occurring fatty acids, producing emulsan analogs with significantly different emulsifying properties.

Lyophilized lipase (10 μg), emulsan (5 mg), and the organic phase (0.25 M caprylic acid, in 2-butanone) were equilibrated (separately) at a_w 0.84 using KCl vapors (Valivety *et al.* 1992). The reactants (1 ml) were combined in 4.5 ml sample vials and incubated for 48 hr at 30°C and 300 rpm. Two types of control experiments were used. Reaction vials containing only LipA were used to verify the absence of emulsan in lipase samples. In addition, fatty acid carryover caused by LipA—octanoic acid interactions were determined. A second negative control consisted of using BSA as a protein replacement for LipA, ensuring all reaction vials contain equal amounts of emulsan and protein. After incubation, experimental units were randomly selected for fatty acid analysis and determination of emulsification properties. Standard emulsification assays were performed as described previously (Leahy 1993).

Prior to fatty acid profiling and emulsification assays, residual (free) caprylic acid was removed from the reaction vials, as follows. The reaction vials were chilled on ice followed by precipitation of emulsan and lipase with cold acetone (95% v/v, -40°C) and allowed to stand for 1 hr at 4°C. Non-covalently bound octanoic acid was removed from the emulsan pellet by three additional washes with cold acetone (3 x 1 ml). Acetone washes were discarded and the pellets dried under a gentle stream of nitrogen prior to fatty acid hydrolysis and methylation (FAMES). Dried pellets were

further processed by FAMES prior to fatty acid profiling or resuspended in 30 mM TIS-HCl pH 8.0 in preparation for emulsification assay.

Fatty acid methyl ester (FAMES) preparation

Hydrolyzed emulsan fatty acids were esterified with methanol using the following protocol. Toluene (1 ml) and internal standard (0.3 mM nonadecanoic acid-C_{19}) were added to the emulsan pellet followed by 3 ml 5% (v/v) HCl in methanol. Sample vials were mixed gently, capped under nitrogen, and incubated at 70°C for 2 hr. Samples were cooled to RT for 5 min, transferred to 10 ml screw cap vials fitted with Teflon caps and 5 ml of 6% $KHCO_3$ added. Hexane (0.75 ml) was added and the reaction mixture vortexed for 10 sec. The emulsion created was broken by centrifugation (2000 rpm, 5 min) and the organic phase transferred by Pasteur pipette to auto sampler vials (ASV). Hexane extraction of esterified fatty acids was repeated and the samples dried under nitrogen. Samples were redissolved in 100 µl hexane, and transferred to ASVs fitted with 200 µl glass inserts and analyzed by GC.

Analysis of emulsan fatty acid profiles

Analysis of methylated fatty acids was performed as described but with modifications to the separation method. The following step profile was employed: initial oven temperature of 50°C for 1 min; 30°C min^{-1} ramp to 200°C, constant for 5 min.; 20°C min^{-1} ramp to 240°C, final temperature for 5 min. Peaks were integrated using the standard integrator and the software supplied by the manufacturer.

Unidentified peaks were not used in the fatty acid comparisons. FAMES methyl ester standards were purchased from Sigma; retention times (min) were as follows: caprylic acid, (5.197); capric acid (6.277); lauric acid (7.326); palmitic acid, (10.222), α-hydroxy lauric acid, (10.283), palmitoleic acid (10.559); β-hydroxy lauric acid, (11.197); stearic acid, (12.530); oleic acid, (12.782); nonadecanoic acid (internal standard, 13.570).

Emulsification assay

Emulsifying activity was determined, as previously described (Rosenberg *et al.* 1979), in 125 ml Erlenmeyer flasks containing 0.1 – 1.0 ml emulsifying agent (5 – 75 μg) in 20 mM Tris-HCl/10 mM $MgSO_4$ (pH 7.2) to a final volume of 7.5 ml. Emulsification substrates (0.1 ml) used were octane, jet fuel (grade JP-8), and an equal volume mixture of hexadecane and 2-methylnaphthalene. Flasks were capped and shaken for 1 hr using a Burnell wrist action shaker (55% duty cycle). Contents of the flasks were transferred to Klett tubes and turbidity measurements taken using a Klett-Summerson colorimeter (Klett Mfg., New York) blanked with distilled water and fitted with a green filter. Dilutions were made using distilled water to keep readings in the range of 30 – 150 Klett units. All measurements were corrected for turbidity caused by hydrocarbon emulsion alone. Emulsan concentrations were determined (as appropriate) from a standard curve prepared in the identical manner, using purified, deproteinated emulsan (Petroferm, Fernandina Beach, FL). One emulsification unit is defined as 100 Klett units.

Fatty acid inhibition of LipA

The effect of free fatty acids on LipA activity was examined by adding dodecanoic (lauric) acid to the assay mixture at various times, before and after the addition of LipA, and by direct incubation with LipA before addition of substrate (*p*NPP). Assays were performed under standard conditions, with the addition of 120 µl stock solution lauric acid (final concentration, 10 µM) to the assay mixture. Direct inhibition of LipA by free fatty acids was performed by incubating LipA (0.75 U, in 30mM Tris-HCl, pH 8) with lauric acid at 30°C for 5 min. prior to assay. Interfacial inhibition of LipA by lauric acid was examined by combining fatty acid and substrate in the reaction mixture prior to addition of enzyme and at various times after addition of lipase.

Emulsifying activity of emulsan and selected proteins

A comparative analysis of the emulsifying activity of emulsan, supplemented with various purified proteins was performed. Emulsifying activity of purified, deproteinated emulsan (Petroferm, Fernandina Beach, FL) was measured with and without the addition of various proteins. Lyophilized emulsan (500 µg) was dissolved in selected protein solutions (20 µg ml^{-1} in 30 mM Tris-HCl, pH 8) and emulsifying activity measured using a 1:1 (v/v) mixture of hexane:2-methylnaphthalene as the substrate. Protein concentration of emulsan was below detectable levels prior to exogenous protein addition as determined by the method of Bradford (Bradford 1976). Emulsan preparations thus formed contained 2% (w/w) protein. Proteins assayed for their effect on emulsification included: BSA, carbonic

anhydrase (Sigma, St. Lousi, MO), cytochrome c (Sigma), *P. fluorescens* lipase (AK "Amano" 20, Amano Enzyme, Lombard, IL), RAG-1 lipase (detergent free), *C. viscosum* lipase (Sigma). Control experiments included emulsifying activity of proteins alone and emulsan without protein addition.

Statistical analyses

Analyses of variance (ANOVA) were performed on all data using software from SAS (version 8.0) with one exception, which was analyzed by Stat-Ease Design Expert (version 5.0.9). In all cases, a natural log transformation of the data was used to satisfy the model assumption of homogeneity of variance.

RESULTS AND DISCUSSION

Synthesis of octyl butyrate

Although LipA is resistant to denaturation in the presence of many organic solvents (Table 3-2), the catalytic activity of the enzyme was not examined in their presence. Activity of lipases in organic solvents has been shown to vary with the polarity of the solvent and total water content of solid (lipase) and liquid phases (Valivety *et al.* 1994). Before examining LipA directed modification of emulsan fatty acids, it was necessary to make an assessment of the catalytic ability of LipA in an organic solvent system. For this purpose, the transesterification (alcoholysis) of vinyl butyrate ester with 1-octanol was selected because of its relative simplicity, favorable thermodynamics, and ease of detecting the reaction products.

The production of octyl butyrate under the conditions described showed that LipA is capable of high catalytic activity in organic media (Fig. 4-1). The data also show lipase-catalyzed transesterification of vinyl butyrate is dependent upon water activity. The highest product concentration (220 μmole ml^{-1}) was detected in experiments equilibrated to a_w 0.86 over KCl salts, suggesting this water content approximates that required for optimal conditions. This figure shows the theoretical maximum product concentration under the conditions described. Experiments equilibrated at higher water content (a_w 0.96) showed decreased activity, suggesting increased competition of the back reaction (hydrolysis). In experiments in which

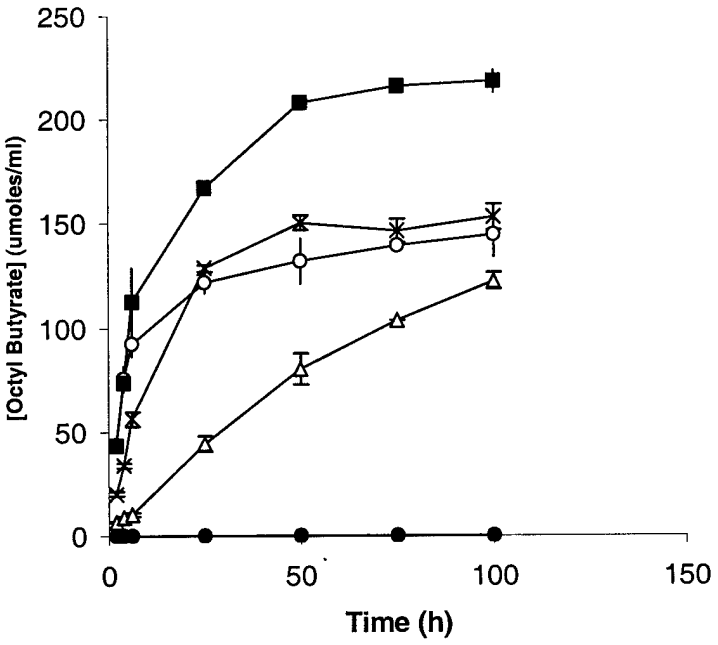

Fig. 4-1. Time course of 1-octyl butytrate synthesis at various a_w. Vinyl butyrate was transesterified with 1-octanol in hexane. The reaction mixtures (lyophilized lipase (10 μg) and liquid phase) were equilibrated to various levels of water activity (a_w) as described in the text: (Δ), a_w 0.33; (○), a_w 0.55; (■), a_w 0.86; (x), a_w 0.97. No product formation was detected in control experiments (●) lacking lipase. Reaction vials were incubated at 40°C and 300 rpm.

system water exceeds a_w 0.55, reaction equilibrium is reached in approximately 48 hr. Assays conducted at a_w 0.33 reveal that equilibrium is not established during the time course of the experiment. This suggests that at water activity levels a_w 0.33 and below, catalytic activity may be limited by the hydration level of the enzyme.

Determinations of initial reaction rates were calculated by fitting product concentrations to a linear function (regression analysis). Initial reaction rates were found to vary with total water content (Fig. 4-2). The highest reaction rate (18.9 μmoles hr^{-1} · 10 μg enzyme) was obtained in experiments in which both phases were equilibrated to a_w 0.86 suggesting this water content is near optimal. At a_w 0.97, the initial reaction rate declined to 8.8 μmoles hr^{-1} · 10 μg enzyme. The decrease in activity may be explained by physical aggregation of individual lipase particles. Poor

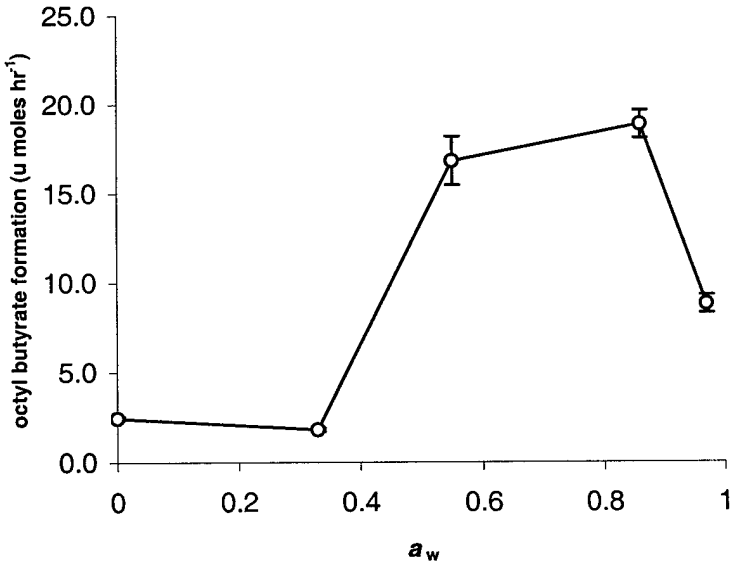

Fig. 4-2. Initial reaction rates of octyl butyrate formation at various a_w. No product formation was detected in control experiments (lacking LipA).

particle dispersion was seen at high water activity (a_w 0.97), but may have occurred to a lesser extent at lower water activity levels. Also, it is highly probable that hydrolysis of vinyl butyrate (forming butyric acid and vinyl alcohol) is significant at a_w 0.97, competing with lipase catalyzed transesterification. Unfortunately, detection of hydrolysis products was not possible, presumably due to interactions of butyric acid with the column matrix. Finally, there may have been some loss of protein rigidity due to excess water, followed by thermal denaturation (Ahern and Klibanov 1985).

Initial reaction velocities shown in Fig. 4-2 are in accordance with those reported for *P. cepacia* under similar conditions (Secundo *et al.* 1999). However, the optimum rate obtained in organic media represents 11% of the activity attained by LipA in aqueous media using *p*NPP as the substrate (not shown). This is not unusual, as similar magnitude of activity loss in nonaqueous media have been reported by others (Zaks and Klibanov 1988; Klibanov 1989). However, the reason for activity loss in hexane is uncertain, but may be due to protein-protein association, resulting in an inaccessibility of the active site, as previously suggested (Zaks and Klibanov 1988). Catalytic activity in this system would presumably be enhanced by immobilization of the lipase to a solid support matrix. Support matrixes often disperse enzyme particles more efficiently (Valivety *et al.* 1994). Increases in activity occurred after adsorption to anion-exchange resin and diatomaceous earth (Celite) by lipases from *Candida cylindracea*, *Aspergillus niger*, and *Pseudomonas fluorescens* (Mustranta *et al.* 1993), and *P. cepacia* (Secundo *et al.* 1999).

Transesterification of emulsan

To determine whether LipA was capable of modifying the emulsan lipophilic component, the covalently attached fatty acids were exchanged for free fatty acids present in the medium. An *in vitro* approach was used, in which the reaction system consisted of LipA, emulsan, and excess molar concentration (250 mM) of octanoic (caprylic) acid in butanone. Reaction components were equilibrated to a_w 0.86. In these experiments, butanone was used in place of hexane because preliminary studies showed an increased solubility of emulsan in this solvent.

Emulsan samples examined prior to the transesterification experiments were found to contain a high percentage of dodecanoic (C_{12}) acids (saturated and hydroxylated), comprising 55% of the total fatty acids (Table 4-1). As expected, octanoic acids were not detected. Emulsan fatty acid substituents (mole %), shown in Table 4-1, compared favorably to those reported by others (Belsky *et al.* 1979; Gorkovenko *et al.* 1997). However, the total fatty acid content (83.7 nmoles mg^{-1} emulsan) was below average, suggesting that this emulsan may not be as active as those purified by others (Belsky *et al.* 1979). Alternately, the apparently low total fatty acid content may be due to incomplete hydrolysis and methylation of *N*-acyl linked fatty acids during FAMES preparation.

Table 4-1. Fatty acid composition of Emulsan

Fatty acid	nmoles/mg emulsan ± SE	mole %
C_8	0	0
C_{10}	4.4 ± 0.38	5.6
$C_{12:0}$	7.6 ± 0.60	9.1
$C_{12,\ 2OH}$	25.2 ± 2.31	28.9
$C_{12,\ 3OH}$	22.7 ± 2.62	26.1
C_{16}	7.3 ± 0.79	8.6
$C_{16:1}$	1.8 ± 0.17	2.4
C_{18}	1.5 ± 0.19	2.0
$C_{18:1}$	4.0 ± 0.33	5.3
Unidentified	9.2 ± 0. 08	12.0
Total	**83.7**	**100**

[1]Emulsan was a gift from Petroferm (formerly Emulsan Biotech, Fernandina Beach, FL)

Changes in emulsan fatty acid profile after incubation in the presence of LipA and octanoic acid are shown in Fig. 4-3. LipA catalyzed octanoic acid substitution of emulsan appeared to occur primarily at the expense of β-hydroxy lauric acid ($C_{12,\ 3OH}$). A 10 % reduction in this constituent is seen in emulsan incubated with LipA. Palmitic acid ($C_{16:0}$) concentration was reduced (3 %) under the same conditions. Emulsan incubated in the presence of LipA contained a concomitant 10 % molar excess octanoic acid over that observed in the control experiments (emulsan + BSA). No fatty acids were detected in experiments containing LipA alone (not shown), verifying the absence of emulsan in the lipase samples. Significant differences in emulsan fatty acid composition were found for β-hydroxy lauric acid (linear model,

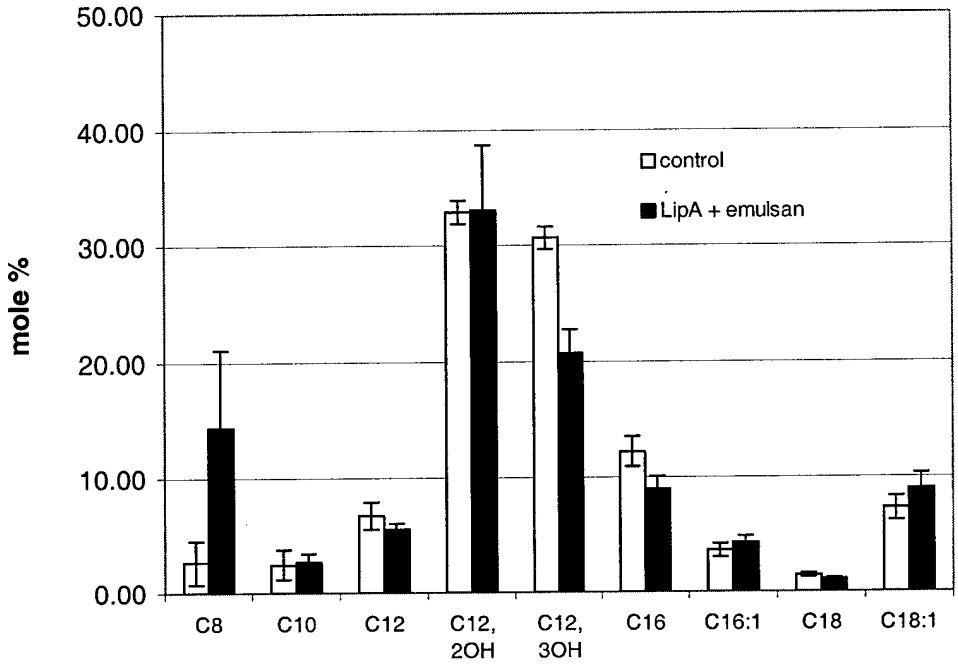

Fig. 4-3. Fatty acid composition of emulsan incubated in the presence of LipA and octanoic acid. Emulsans were equilibrated to a_w 0.86 and incubated in butanone for 48 hrs (30°C) in the presence of LipA or BSA (control) equilibrated to the same a_w prior to analysis by FAMES. Values are the mean of at least five replications.

two-stage nested design, $p < 0.01$) and palmitic acids ($p \leq 0.05$). No other significant differences in fatty acid composition were found. These results suggest that the $C_{12,\ 3OH}$ and $C_{16:0}$ acids are preferentially replaced by octanoic acid in LipA—catalyzed transesterification. However, the data clearly showed small quantities of octanoic acid were detected in the control experiments. Assuming detectable quantities of non-enzymatic transacylations do not occur, the presence of octanoic acid in the control experiments indicates residual quantities remain after acetone washing. More rigorous washing eliminated this problem but at the cost of reduced yields. Further, the data showed a high degree of variability, with respect to octanoic acid

incorporation. The source of this variation may be attributed to the washing procedure, but also suggest the reaction conditions are more dynamic than originally assumed. Random variability within the groups (residual error) showed evidence of non-random scatter (bell-shaped trend). To account for this, a natural log transformation was taken for all data. The analysis was recomputed and the groups found to show evidence of random scatter. Analysis of transformed data showed no significant difference between emulsans, with respect *n*-octanoic acid incorporation ($p=0.35$).

Based on the results of previous experiments, RAG-1 lipase could be expected to show high catalytic activity under the conditions described (Fig. 4-1). Therefore, it is plausible that enzymatic transesterifications did not occur to a significant degree due to the nature of the substrate itself. The highly branched polysaccharide backbone of emulsan may limit acyl exchange by preventing the catalytic site from accommodating the requisite fatty acid. Changes in substrate specificity of dried enzymes have been reported by others and attributed to increased protein rigidity (caused by drying) resulting in failure to accommodate bulky substrates (Zaks and Klibanov 1984). Although some evidence for LipA-catalyzed transesterification is presented, the lack of a significant difference in octanoic acid composition suggests the reaction is not favored under the conditions examined. In addition, it weakens the argument that LipA is highly active in this regard and suggests an alternative role for the enzyme.

Emulsification properties of tailored emulsans.

Emulsan samples were harvested from transesterification reaction vessels by acetone precipitation, washed, resuspended in buffer and assayed for emulsifying activity. The protein component of reaction vessels was also precipitated by this method and therefore was present in emulsan samples. The emulsifying activity of transesterifed emulsans (and protein) was examined using octane, jet fuel, and a 1:1 mixture of hexane and 2-methylnaphthalene as substrates (Fig. 4-4). Emulsans incubated in the presence of LipA showed significantly better emulsifying activity

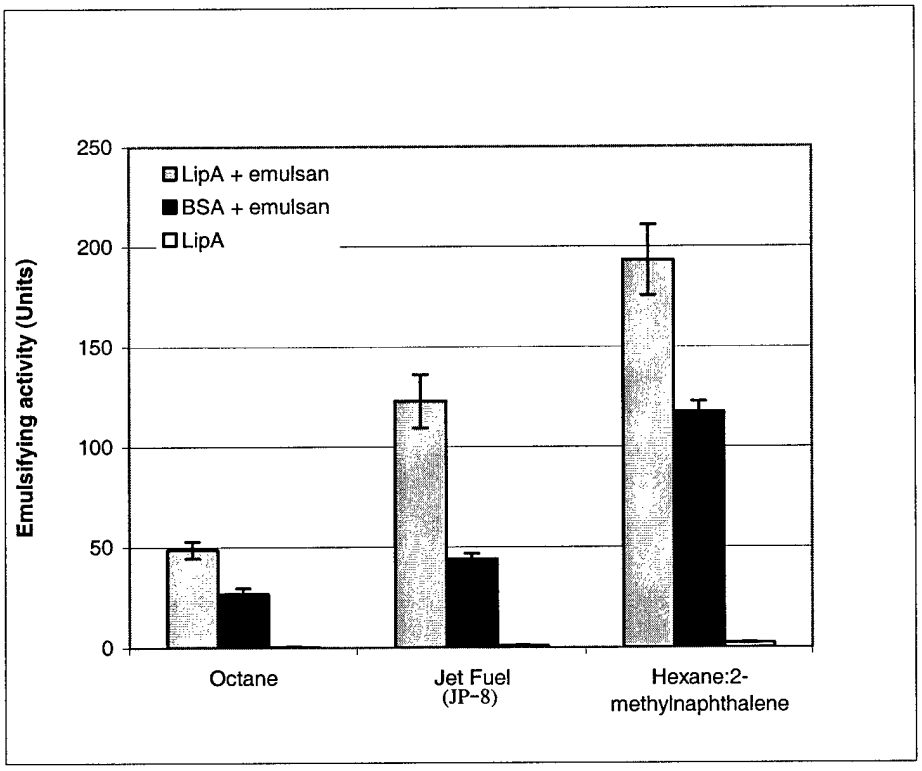

Fig. 4-4. Emulsification properties of emulsans incubated in the presence of LipA and octanoic acid. Emulsans (5 mg) were equilibrated to a_w 0.86 and incubated in butanone for 48 hrs (30°C) in the presence of 10 µg LipA or BSA (control) equilibrated to the same a_w prior to assay. Reaction mixtures were both precipitated and washed with acetone prior to assay. Values are the mean of at least five replications ± SE.

compared to those incubated with the equivalent quantity of BSA (linear model, two-stage nested design, $p<0.01$). Emulsan incubated with LipA and octanoic acid showed approximately 80%, 180%, and 65% increase in activity over control experiments using octane, jet fuel, and hexane/methylnaphalene substrates, respectively ($p<0.01$). Experiments containing LipA alone showed no emulsifying activity. Increased emulsifying activity of transesterified emulsan toward octane was expected, if LipA-catalyzed transfer of octanoic acid was successful. However, we expected native emulsan to have higher emulsifying activity toward the remaining substrates, as its long-chain fatty acid substituents could be expected to interact more strongly to long-chain alkanes present in those substrates. The presence of octanoic acid on transesterified emulsans was expected to decrease emulsifying activity against jet fuel and hexane/methylnaphthalene substrates. This result was not observed. Emulsan incubated with LipA showed higher emulsifying activity against all substrates than emulsan incubated with BSA. Considering the low percentage change in fatty acid composition (i.e., octanoic acid increased to 14.5 mole %, Fig. 4-3), the significant increase in emulsifying activity of LipA-emulsan mixtures is presumably not a result of octanoic acid addition to the polymer. Instead, these data suggest the presence of LipA itself enhances emulsifying activity. Therefore, the data does not support the hypothesis that successful lipase-catalyzed acyl transesterification of emulsan occurred resulting in a concomitant change in emulsifying activity. Further, they suggest the presence LipA in emulsan samples provides an additive effect to emulsifying activity above that observed in samples containing BSA.

Comparative emulsifying activity of emulsan and selected proteins

The possibility that a specific emulsan-LipA association exists that may enhance the ability of the polymer to stabilize emulsions was investigated. Emulsan samples were dissolved in various protein solutions and assayed for emulsifying activity (Fig. 4-5). The data showed that LipA addition to emulsan significantly increased emulsifying activity (one-way ANOVA, multiple comparisons calculated using Bonferroni adjustment, alpha = 0.05) more than 2-fold (22.9 U ± 0.6) from that observed in emulsan without protein (11.1 U ± 0.6). LipA (no emulsan) had little emulsifying activity (2.0 U ± 0.2). These results demonstrate the synergistic effect of

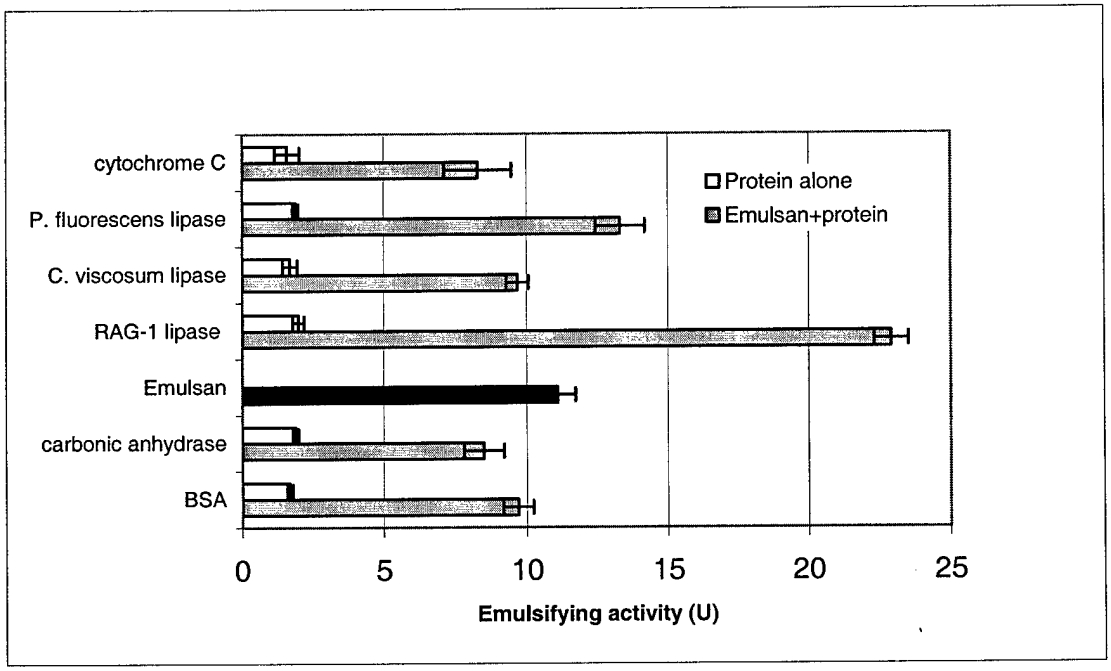

Fig. 4-5. Emulsifying activity of RAG-1 lipase and emulsan. Emulsan (500 μg) was dissolved in protein solutions (20 μg ml^{-1} in 30 mM Tris-HCl, pH 8) and assayed for emulsifying activity using hexane:2-methylnaphthalene as the substrate. Emulsifying activity of emulsan without protein addition, (dark bar, —) and individual proteins was also determined. Data are the means of four replications ± SE.

LipA addition to emulsan, as emulsifying activity increased 1.7-fold over the predicted value derived from the sum of individual components (13.1 U). The least significant difference (LSD) test showed emulsan samples incubated with *P. fluorescens* lipase showed a 20% increase in emulsifying activity (alpha = 0.05) suggesting it shares some characteristics with LipA that enhance emulsifying activity.

The fact that LipA showed little emulsifying activity was of particular interest, as proteins associated with other polymeric emulsifiers have been reported to possess emulsifying properties. Toren et al. (Toren *et al.* 2001) showed the bioemulsifier purified from *Acinetobacter radioresistens*, referred to as alasan, is a high molecular weight complex of polysaccharide and protein. Three alasan proteins were isolated from the polymer and shown to be the active emulsifying components of the alasan complex (Toren *et al.* 2001). It is apparent from the data presented, that LipA does not show emulsifying activity in the manner of alasan proteins. Alternate mechanisms for enhancing emulsifying activity are suggested. LipA may interact with the lipophilic moiety of the polymer, causing a conformational change in its structure. This may allow more polymers to colonize the oil-water interface or enhance their attachment. LipA association with emulsan may also decrease oil droplet coalesence, increasing emulsion stability.

Fatty acid inhibition of LipA

Hydrophobic interaction between emulsan fatty acids and LipA provides a plausible mechanism for association between lipase and emulsifier. The combination

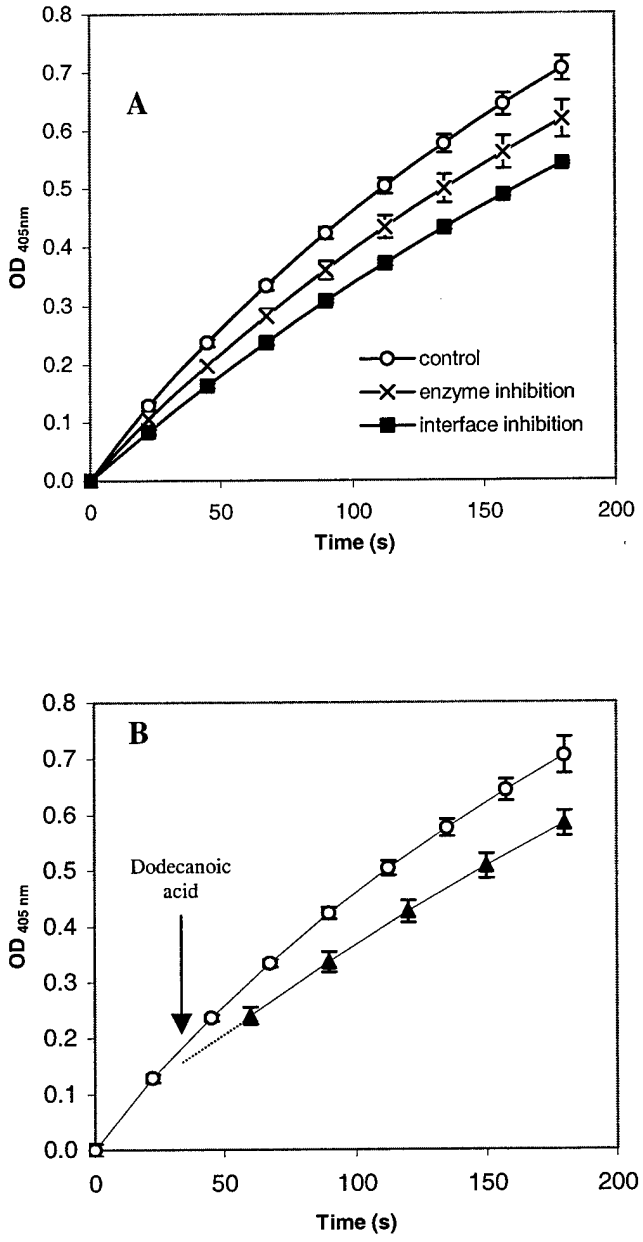

Fig. 4-6. Dodecanoic acid inhibition of LipA. (A). Dodecanoic acid (10 μM, final concentration) was added to assay components at various times: (x), enzyme inhibition: LipA incubated with free acid prior to assay; (■), interface inhibition: lauric acid added to substrate before LipA; (○), control (no addition). (B) Lauric acid added in the presence of active LipA: (▲), after 30 seconds reation time; (○), control (no addition);(dashed line), hypothetical.

of these two components results in higher emulsifying activity than either component alone (Fig.4-5). It is probable that this association also occurs in culture supernatants where LipA showed high lipase activity (Fig. 2-1). These data lead to the prediction that, despite the presence of emulsan fatty acids, LipA retains high lipase activity as it functions both as a lipolytic enzyme and emulsan-associated protein. To examine the effect of fatty acids on LipA, dodecanoic (lauric) acid was added at selected intervals to assay mixtures containing LipA and *p*NP in phosphate buffer prior to determination of lipase activity. Lipase inhibition by lauric acid and interfacial activity has been demonstrated previously (Markweg-Hanke *et al.* 1995).

The rate of product formation was decreased 10% when LipA was incubated in the presence of lauric acid (Fig. 4-6A). Although the rate of product formation is significantly different from control experiments (repeated measures analysis, $p<0.05$), the low level of inhibition did not seriously affect product formation. This result suggests lauric acid does not act through competitive inhibition, and/or is easily displaced from the hydrophobic active site during substrate binding. Further, it supports the hypothesis that lipolytic activity remains high in the presence of emulsan fatty acids. However, when lauric acid was allowed to occupy the substrate-water interface before LipA, over 20% reduction in the rate of product formation was observed ($p < 0.01$). This indicated that the presence of free fatty acids modulates the adsorption of lipase molecules to the interface, although overall catalytic rates remain high. Lipase already active in the reaction mixture was not significantly inhibited by lauric acid (Fig. 4-6B). Lauric acid, added after 30 seconds of reaction time, did not significantly decrease the reaction rate (analysis of covariance to compare two

slopes). Presumably, enzyme molecules that were already absorbed to the interface were protected from inhibition. Taken together, these data indicate lipase activity is not adversely affected by the presence of free fatty acids and that LipA should retain nearly all activity as part of an emulsan-LipA complex.

Chapter summary

Data presented here show LipA retains high catalytic activity in organic media. Transesterification reaction rates determined in hexane compared favorably with those reported for other lipases, most notably, *P. cepacia* lipase (Secundo *et al.* 1999), a widely used biocatalyst in non-aqueous media and commercial product (Svendesen *et al.* 1995; Amano Enzyme Inc. 2000). LipA showed the highest initial reaction rate at a_w 0.86, indicating this water content approximates the optimum for the reation. Under these conditions, LipA synthesized the near maximum theoretical concentration of product.

Despite high activity observed in organic solvent, we were unable to conclusively demonstrate that RAG-1 lipase serves as the biocatalyst for transacylations of emulsan fatty acids. Although significant differences in the two fatty acid components were observed, changes, with respect to the transesterified fatty acid, were not significant. It is concluded that the bulky nature of the emulsan polysaccharide backbone prevents proper accommodation of the fatty acyl chains. Identification of this role for LipA may be possible under different experimental conditions.

Emulsifying activity of emulsan increased in the presence of LipA. We found a greater than 2-fold increase in emulsifying activity when emulsan was mixed with

RAG-1 lipase. This result suggests that there is a LipA-emulsan specific association responsible for the increased emulsifying activity observed. This may occur by a lipase-induced conformational change in emulsan structure, or by preventing oil droplet coalescence. It is suggested the two components may associate by hydrophobic interaction between the lipase and lipophilic fatty acids.

LipA remained highly active in the presence of dodecanoic acid. Only limited inhibitory effects of the fatty acid were noted. The highest degree of inhibition (20%) occurred at the interface, purportedly by blocking LipA access to the substrate. Interfacial inhibition was reduced if the lipase had access to the substrate prior to fatty acid addition. These data support the hypothesis that LipA retains almost complete activity in the presence of emulsan fatty acids and that any interaction between the two components does not diminish its activity as a lipolytic enzyme.

Chapter 5. GENERAL DISCUSSION AND CONCLUSIONS

Throughout this work, two major objectives were pursued. The first was to establish the role of lipase in modifying the fatty acid moiety of emulsan. Previous research in our laboratory has suggested that the extracellular lipase produced by RAG-1 may be an acylating agent responsible for attaching n-alkanoic acids to the emulsan polymer (Leahy 1993). This hypothesis has been supported by results of other research in which an acylating agent(s) had been proposed (Gorkovenko *et al.* 1997). Our objective was to establish LipA as the acylating agent and determine the conditions under which transacylation of the emulsan polymer is possible. This objective supports a larger goal of expanding the industrial applications of the biopolymer by tailoring its chemistry, creating emulsan analogs with unique properties. The second objective was to understand the properties of LipA itself, in order to assess its potential for use in biotechnology. For this purpose, the lipase was purified and its properties examined. Today, industrial enzymology is a rapidly expanding field of which lipases comprise a significant portion. Industrial lipases differ with respect to reaction rates, substrate specificity, stereo-specificity, pH, and temperature optima in aqueous and non-aqueous media. The search for new enzymes with unique properties has intensified as applications expand or new ones are envisioned (Arbige and W.H. Pitcher 1989).

Potential of LipA as an industrial biocatalyst

We reported here the purification and properties of the extracellular lipase, LipA, produced by *Acinetobacter* sp. RAG-1. RAG-1 cells were found to produce

large quantities of lipase when grown on hexadecane. The lipase is stable in this medium and showed no apparent loss of activity at 30°C. The enzyme was hydrophobic in character and showed some tendency toward aggregation. LipA showed high activity against long-chain triglycerides and is, therefore, considered to be a true lipase.

The basis for arguing that there is an enormous biotechnological potential for lipases is broad, namely that they are (1) stable in organic solvents, (2) demonstrate broad substrate specificity, and are (3) co-factor independent, and (4) show high enantioselectivity (Jaeger and Reetz 1998). The data reported here clearly demonstrate RAG-1 lipase possesses many of these characteristics. LipA was stabile in most of the organic solvents examined (Table 3-2). Moreover, lyophilized LipA remained highly active in organic media, even under nearly anhydrous conditions (Fig. 4-1). Initial reaction rates for the transesterification of *n*-octanol with vinyl butyrate were of the same magnitude as those reported for crude *P. cepacia* lipase, a commercially available product (Secundo *et al.* 1999). High activity in non-aqueous media may be the single, most important characteristic of RAG-1 lipase as applications for lipasest in organic media are expanding rapidly (Jaeger *et al.* 1999b). Moreover, the initial reaction rates observed can reasonably be expected to increase with lipase immobilization onto a solid support (Reslow *et al.* 1988; Koops *et al.* 1999). While cofactor independence was not unequivocally demonstrated, it may be reasonably assumed for all lipases (Jaeger and Reetz 1998; Arpigny and Jaeger 1999). Stereo-selectivity of RAG-1 lipase remains to be demonstrated (4). Resolution of enantiomers of primary and secondary alcohols and aymmetric synthesis are common

industrial applications of lipases (Amano Enzyme Inc. 2000) and should be the focus of future research on RAG-1 lipase.

Other properties of RAG-1 lipase make it an excellent candidate for further study. These properties include activity at high temperatures, pH tolerance, stability in the presence of Ca^{+2}, activity in the presence of many inhibitors, and resistance to denaturation by metals (Fig 3-4, Table 3-1). These characteristics also suggest LipA should remain highly active in petroleum polluted environments resembling that from which RAG-1 was originally isolated. Properties of LipA that may limit applications of the lipase are its loss of activity in the presence of EDTA and inactivation by pyridine. Chelating agents are commonly used in detergent formulations (Rao *et al.* 1998) but substitution of pyridine is easily achieved. It should be noted that some industrial lipases share these short comings (Bozoðlu *et al.* 1984; Svendsen *et al.* 1995).

Role of RAG-1 lipase in emulsan transesterification

A cell-free system was designed to test the hypothesis that LipA functions in trans acylation (transesterification) of emulsan. In this system, purified LipA was incubated with emulsan and *n*-octanoic acid in butanone and the resultant emulsan fatty acid composition was analyzed by gas chromatography. All system components were equilibrated to optimum a_w, as previously established (Fig. 4-2). Although the data showed a reduction in mole % of two emulsan fatty acids after incubation in the presence of LipA, it cannot be concluded with confidence that a concomitant percentage increase of octanoic acid was observed (Fig. 4-3). Since LipA had high

activity in non-aqueous media, it is proposed that other factors prevented efficient trans acylation of emulsan fatty acids. It may be that the bulky polysaccharide backbone of the emulsifier prevents adequate fit of fatty acids and *n*-octanoic acid inhibiting catalysis. Alternately, substrate specificity changes occurring during drying of enzymes have been reported by others (Zaks and Klibanov 1984). Following this rationale, the fact that RAG-1 lipase is most active against acyl chain lengths of $C_6 - C_8$ in aqueous solution (Fig. 3-3) may not dictate similar specificity under non-aqueous conditions. Repeating the experiments using longer-chain alkanoic fatty acids as acylating agents may resolve this question.

Conclusively demonstrating LipA-transesterification of emulsan fatty acids requires further work. Based upon the data presented here, future research should focus on optimizing experimental conditions. Specifically, examining substrate specificity of lyophilized lipase, solvent selection, and lipase immobilization are all elements that may be improved upon to produce desired results. The ability to bioengineer emulsan analogs through enzyme catalysis remains attractive. High stereo-selectivity of lipase catalysts may provide modification of the bioemulsifier not possible through chemical means or by selected feeding strategies.

RAG-1 lipase and emulsification production

As part of transesterification experiments, emulsifying activity of acylated emulsans was determined (Fig. 4-4). Results showed significantly higher emulsifying activity for emulsan incubated in the presence of LipA and octaonic acid over the control, despite inconclusive evidence of successful LipA-catalyzed

transesterification of the polymer. The result stimulated interest in the possible enhancements LipA may provide to emulsan activity. Selected proteins were incubated with emulsan and emulsifying activity of the mixtures was determined (Fig. 4-5). The data indicate that the presence of LipA increased emulsifying activity of emulsan more than 2-fold, suggesting association between lipase and emulsifier. The combination of LipA and emulsan increased emulsifying activity more than could be predicted from the sum of the components. Although the purity of LipA preparations (judged by SDS-PAGE) showed little or no contamination (Fig. 2-4), the presence of other components in lipase samples being the active agent cannot be totally ruled out. However, the presence of residual detergent (Triton X-100) or natural surfactants can be excluded by the absence of emulsifying activity in the lipase samples (Fig. 4-5). Further, the presence of resdual emulsan or free fatty acids were not detected in lipase samples analyzed by gas chromatography (not shown).

Native emulsan is known to be a complex of amphiphilic polysaccharide and protein (Zuckerberg *et al.* 1979). While the polysaccharide and covalently bonded fatty acids have been well characterized (Zuckerberg *et al.* 1979; Rosenberg *et al.* 1979; Belsky *et al.* 1979), no emulsan-associated protein was identified. This is surprising, considering the importance of protein in emulsification (Zosim *et al.* 1987). Similar polysaccharide-protein complexes have been demonstrated in other polymeric emulsifiers, including alasan from *A. radioresistens* (Navon-Venezia *et al.* 1995) and *A. calcoaceticus* BD4 emulsan (Kaplan *et al.* 1987). Covalently bound protein has been shown to be the active emulsifier in alasan (Toren *et al.* 2001), while a non-covalent association is required for emulsifying activity of the BD4 emulsan.

These emulsifiers lose activity when separated from their associated proteins (Kaplan *et al.* 1987; Toren *et al.* 2001).

Our data indicate that RAG-1 emulsan may be different from other polymeric emulsifiers in that absence of a protein component does not inactivate the emulsifier. Emulsan, without associated protein, remained active (Fig. 4-5). Presumably, covalently-bound fatty acids in RAG-1 emulsan, alone, are sufficient to bind to the interface and stabilize the emulsion (Belsky *et al.* 1979; Zuckerberg *et al.* 1979). However, LipA addition to RAG-1 emulsan produced a greater than 2-fold increase in emulsifying activity over that observed in controls, suggesting the lipase functions as an emulsan-associated protein (Fig. 4-5). Other proteins examined did not increase emulsifying acitivity, indicating the effect is LipA specific. In support of this hypothesis, we found that high lipolytic activity was associated with native emulsan prepared from crude supernatants by ammonium sulfate precipitation (Zuckerberg *et al.* 1979) and putative LipA protein in those precipitates separated by SDS PAGE (data not shown).

Proposed model for RAG-1 lipase as an emulsan-associated protein

A model was developed, to elucidate the effects of RAG-1 lipase on the structure and activity of native emulsan (Fig. 5-1). The two components comprise a polysaccharide-protein complex and are presumed to associate via hydrophobic interaction. This is not an unreasonable assumption, as many hydrophobic

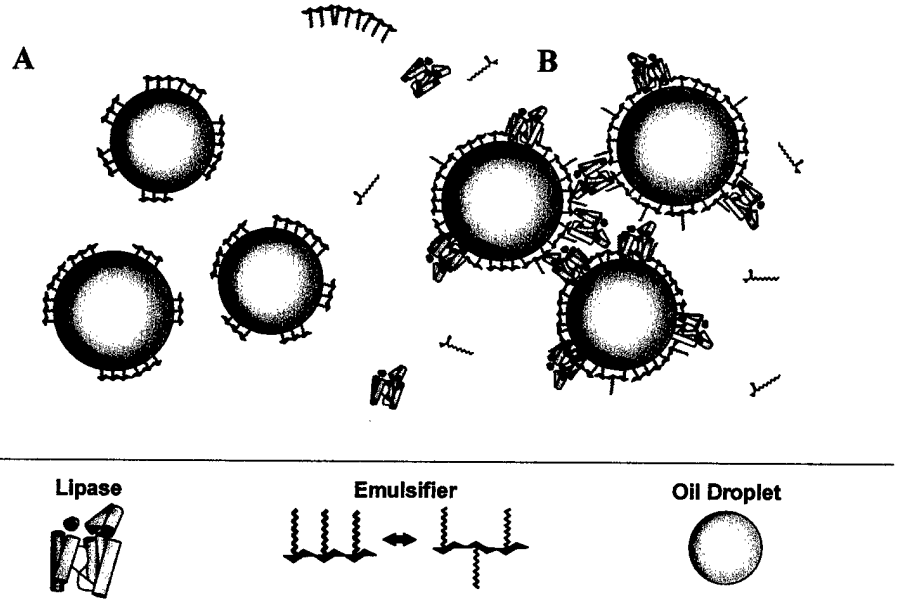

Fig. 5-1. LipA as an emulsan-associated protein. (A) Emulsan, in the absence of LipA, colonizes the oil-water interface preventing droplet coalescence. (B) Hydrophobic interaction allows association of emulsan fatty acids and LipA to form a polysaccharide-protein complex. The lipase component increases emulsifying activity by promoting conformational change in the polymer allowing more molecules to colonize the interface. In addition, the protein component provides bulk to the complex limiting droplet coalescence. Unbound emulsan and free fatty acids are also shown.

(Breuil and Kushner 1975b; Kordel *et al.* 1991; Bompensieri *et al.* 1996) or amphiphilic (Jaeger *et al.* 1992) lipases have been reported. In addition, hydrophobic interactions between lipases and dyes have been demonstrated (Markweg-Hanke *et al.* 1995). Our data suggest that LipA is also hydrophobic, since detergent was required to elute the lipase from HIC matrixes (Fig 2-3).

Alternate hypotheses are that LipA may bind emulsan fatty acids at the hydrophobic catalytic site or via a fatty acid specific, non-catalytic site. However, these hypotheses are not as attractive as hydrophobic interaction. Presumably,

binding of emulsan fatty acids at the catalytic site would be detrimental to lipolysis. We found that RAG-1 lipase remained highly active in the presence of fatty acids, indicating they do not occupy the catalytic site (Fig. 4-6). Further, the presence of a non-catalytic binding site is not supported by three-dimensional structures elucidated by X-ray crystallography. We predict that increased emulsifying activity would also be observed in LipA-emulsan complexes if the catalytic site of LipA were bound irreversibly by a non-hydrolysable acyl glycerol analog (i.e., 1, 2-dicotylcarbamoyl-glycero-3-*O*-*p*-nitrophenyl octyl phosphate).

Native emulsan, complexed with LipA and other hydrophobic proteins, may exist in a more compact form. Compacting the branched polysaccharide allows accumulation of more complexes at the oil-water interface, adding increased stability to the emulsion (Fig. 5-1). The presence of LipA may also limit oil droplet coalesence by adding bulk to the polymer. Observing changes in emulsan viscosity or interfacial tension after addition of LipA should permit a good test of the model. The presence of LipA in "purified" emulsan samples may be confirmed by Western blotting. Such findings, with the data accumulated in this study, would provide strong evidence for LipA being one of the active protein components, if not the active component, in native emulsan.

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